UNIVERSIDADE ESTADUAL DE CAMPINAS

Valdemar Antonio Paffaro Junior

CARACTERÍSTICAS FENOTÍPICAS E FUNCIONAIS DAS CÉLULAS *NATURAL KILLER* UTERINAS DE CAMUNDONGOS

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) VALDEMAR ANTONIO PAFFARO JUNIOR

e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na área de Histologia.

Orientador: Prof.Dr. Áureo Tatsumi Yamada

Co-Orientador: Prof.Dr. Paulo Pinto Joazeiro

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP

P14c

Paffaro Júnior, Valdemar Antonio
Características fenotípicas e funcionais das células *Natural Killer* uterinas de camundongos/Valdemar Antonio Paffaro Júnior. -- Campinas, SP:[s.n.], 2002.

Orientador: Áureo Tatsumi Yamada
Co-orientador: Paulo Pinto Joazeiro
Tese (Doutorado) – Universidade Estadual de Campinas. Instituto de Biologia

1.Reprodução. 2.Linfocitos. 3.Utero. I. Yamada, Àureo Tatsumi. II. Joazeiro, Paulo Pinto. III.Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Data da Defesa: 31/01/2003

BANCA EXAMINADORA

Prof.Dr. Áureo Tatsumi Yamada (Orientador) — Assinatura

Prof.Dr. Fernando Ferreira Costa — Assinatura

Profa.Dra. Maria Angelica Miglino — Assinatura

Prof. Dr. Paulo Maria Ferreira de Araújo — Assinatura

Prof.Dr. Paulo Alexandre Abrahamsohn — Assinatura

Prof.Dr. Luís Antônio Violin Dias Pereira — Assinatura

Profa.Dra. Maria Celia Jamur — Assinatura

Dedico este trabalho,

Á minha esposa e filhos, Andréa, Bruno e Tiago,

Pela compreensão, amor e carinho, até mesmo nos momentos em que estivemos distantes.

Aos meus pais Valdemar e Ondina,

Pelo amor, carinho e ensinamentos valiosos que jamais encontraria nos livros.

À minha irmã Kelly,

Pela amizade e pelo exemplo de força para alcançar seus objetivos.

Agradeço a Deus,

Pelo maravilhoso milagre que é a vida.

AGRADECIMENTOS

Ao Prof. Áureo T. Yamada, pela amizade, orientação segura deste trabalho e pelo exemplo de seriedade, dedicação e profissionalismo que nortearam grande parte de minha formação acadêmica e profissional.

Ao Prof. Paulo P. Joazeiro, pela amizade, pela co-orientação deste trabalho e pelos comentários e sugestões sempre pertinentes ao nosso estudo

À Prof. Anne Croy, pela maneira que me acolheu em seu laboratório, dando toda atenção e incentivo que propiciaram a execução de parte de nossos trabalhos.

Ao Professor Luiz Antonio Violin Dias Pereira, Prof. Maria do Carmo Alberto Rincon e Prof Carla B. C. Buzato, pela agradável convivência e apoio.

Aos docentes do Departamento de Histologia e Embriologia do IB/UNICAMP, pela oportunidade de aprendizado.

Ao amigo Gordon Black, pelo apoio para realização de parte deste trabalho, e por propiciar meu rápido entrosamento no laboratório e na cidade de Guelph.

Ao Dr. Hong He, por ensinar os procedimentos de biologia molecular e pela inestimável cooperação em parte de nossos trabalhos.

Ao curso de pós-graduação em Biologia Celular e Estrutural. IB. UNICAMP, por permitir acesso ao conhecimento.

À CAPES, pela bolsa de mestrado, à FAPESP, pelas bolsas de mestrado e doutorado e por propiciar o estágio no exterior e à FAEP/UNICAMP, pelos auxílios.

Aos funcionários do DHE/IB/UNICAMP, Baltazar, Marta, Martinha, Raquel, Cleuza, pelo auxilio técnico e convivência

Às secretárias do DHE/IB/UNICAMP, Rita e Beatriz e do DBC/IB/UNICAMP, Lílian e Silvia que muito facilitaram a realização deste trabalho.

Às minhas amigas Marcia e Suzana, pela amizade e por estarem sempre dispostas a ajudar em qualquer dificuldade.

Aos amigos de departamento, Cristiane, Fabi, Débora e Alex, Patrícia, Silvane, Eliane, Celina, Petra, Elisa, Carolina, Érika, Luciana, Luciana de Santis, Willian, Gustavo, Renata, Priscila, Monique, Aline, Thomaz, pela amizade e agradável convivência dos últimos anos.

Aos meus amigos, Marcus e Gustavo Thomazine, pela amizade, bom humor e por estarem sempre prontos a ajudarem.

Aos colegas, alunos, técnicos e professores do "Biomedical Science Institute. OVC. University of Guelph, Canada", pela convivência, calorosa recepção e colaboração para realização de parte deste trabalho.

Aos camundongos que juntamente com suas crias tiveram suas vidas sacrificadas para a realização deste trabalho.

Aos laboratórios de Citoquímica e Imunocitoquímica e Embriologia Experimental e Criométodos do DHE. IB. UNICAMP, onde foi realizada grande parte deste trabalho.

Ao Laboratório da Prof. B. Anne Croy da "University of Guelph", onde foi realizada parte deste trabalho.

RESUMO

As células *natural killer* uterinas (NKu) constituem uma população linfocitária transitória, presente no útero de humanos e roedores durante a gestação. O ambiente uterino, no início da gestação, parece ser determinante na migração e diferenciação, assim como, na supressão da atividade citolítica destas células, que atuam em outras localidades na resposta imune inata. Porém, os fatores ou os mecanismos envolvidos nestes processos celulares, das NKu, não foram ainda elucidados. As características fenotípicas e constitutivas asseguram a similaridade das NKu com as NK circulantes (NKc), porém, diversos ensaios apontam a primeira como sendo uma provável subclasse de células NK. No presente estudo, estabeleceu-se um método citoquímico utilizando-se a lectina DBA (*Dolichos biflorus*) como um marcador altamente seletivo para as células NKu de camundongos, que permite distingui-las das células NK circulantes e das demais populações linfocitárias, por não apresentar reatividade cruzada. Com este marcador, foi possível identificar morfologicamente os subtipos correspondentes aos estágios de maturação, bem como, estabelecer de forma precisa a incidência e localização das NKu no útero ao longo da gestação. Ao contrário dos imunomarcadores utilizados até então, que apresentavam limitações (restritas a algumas linhagens de camundongos, ou que reconheciam apenas as formas maduras ou imaturas), a lectina DBA mostrou ser um marcador universal para as células NKu de camundongos, além de reagir com formas imaturas e plenamente diferenciadas destas células.

Com o intuito de se elucidar a ação do estrógeno diretamente sobre as células NKu, utilizou-se animais geneticamente modificados juntamente com métodos imunocitoquímicos e moleculares para identificar a possível expressão de receptores de estrógenos (ERα e ERβ) nestas células. A medula óssea de animais deficientes em receptores de estrógenos α e β (ER-KO αe β) foi transplantada em camundongos imunodeficientes B6-Rag-$2^{-/-}/\gamma c^{-/-}$ (T^-,B^-, NK^-). O isolamento e

purificação das células Nku pelo método lectina DBA-biomagnético, tornou-se possível após ter sido constatado que a mesma reage de forma específica com o glicoconjugado presente na superfície das células NKu. Os testes de RT-PCR realizados com as NKu isoladas demonstraram a ausência de receptores de estrógeno α e β nas células NKu de camundongos, revelando que o hormônio estrógeno não seria o indutor que atua diretamente na migração, proliferação ou diferenciação destas células no ambiente uterino

Com o fito de investigar se as NKu constituem um subtipo especializado de células NK, avaliou-se as expressões dos genes de 14 receptores LY49 e do DAP12, através do RT-PRC em tecidos mesometriais dissecados de camundongos virgens, prenhes, normais e geneticamente modificados deficientes em linhagens de linfócitos. Úteros virgens de C57Bl/6J (B6), *randombred* (predominante H-2^b) *scid/scid*, B6-IL-$15^{-/-}$ e B6 *nu/nu* expressaram DAP12 e um repertório parcial de Ly49. A prenhez induziu a expressão de receptores inibitórios adicionais em todas as linhagens estudas. No útero de camundongo B6 virgem os receptores de ativação LY49D e LY49H foram co-expressos com os inibitórios LY49 I, J e Q. No início da gestação (dg6), foi observada a expressão de genes para um repertório extenso de Ly49, que prossegue até a metade da gestação, os quais devem estar relacionados com a diferenciação e comportamento das NK no útero.

O conjunto destes experimentos e os respectivos resultados foram organizados em três capítulos distintos, redigidos na forma de três artigos científicos já submetidos para publicação em revistas especializadas da área.

Saliente-se que partes deste trabalho foram desenvolvidas no laboratório da Profa. Dra. Bárbara Anne Croy, University of Guelph, ON, Canada.

ÍNDICE

CAPÍTULO- I

1- INTRODUÇÃO

No útero de roedores e humanos, durante a gestação, é observado um grande influxo transitório de linfócitos. Estudos de imunofenotipagem em roedores e humanos revelaram que estas células assemelham-se as células *Natural Killer* circulantes (NKc) (Bernard et al, 1978; Mukhtar, et al, 1989; Readline e Lu, 1989; Linnemeyer e Hamilton, 1990; King e Loke, 1991; Croy e Kiso, 1993; Head, 1996; Loke e King, 2000). Em camundongos, a caracterização inequívoca de que estas células uterinas pertencem à linhagem linfocitária NK (NKu) foi obtida através de uma série de experimentos, utilizando diversos animais geneticamente modificados imunodeficientes, transplantados com células de órgãos linfóides primários e secundários (Croy e Kiso, 1993; Guimond et al, 1997; Croy et al, 2000; Greenwood et al, 2000; Chantakru et al, 2002,). Em humanos, as células NK CD56+, $CD16^-$, $CD3^-$ parecem ser análogas às células NKu de camundongos, e representam mais de 70% dos leucócitos encontrados durante a fase secretória do endométrio e na decidua durante a fase inicial da gestação (Loke e King, 2000).

Apesar das comprovações experimentais da origem extra-útero destas células, a partir de órgãos linfóides secundários (Chantakru et al, 2002) e que migram para o útero transitória e exclusivamente durante a gestação (Stewart e Peel, 1980; Peel, 1989; Delgado et al, 1996), não se conhece quais mecanismos ou fatores induzem esta migração para o ambiente uterino, assim como, quais seriam os fatores que induzem a sua proliferação e diferenciação neste ambiente.

Quanto à atividade funcional destas células na gestação, a única comprovada experimentalmente é a síntese e liberação do interferon-gama (Ashkar e Croy, 1999; Ashkar et al, 2000), tendo esta, uma importante ação na vascularização, e na manutenção da decídua e da placenta fetal. Se o papel funcional das células NKu na gestação restringe-se, ou não, apenas à produção de

citocinas para a manutenção do ambiente uterino, propícia ao desenvolvimento embrionário e fetal, é uma questão polêmica. Em se tratando de uma população celular que, apresenta todo o arsenal de substâncias citolíticas nos seus grânulos descritos para as NKc (Peel, 1989; Parr et al., 1990; Croy et al., 1997[a]), por conseguinte, compõem também os quesitos necessários para atuarem potencialmente na resposta imune do tipo inata. No entanto, não há relatos que comprovem a atuação das NKu em atividade citolítica, o que torna intrigante a compreensão de quais mecanismos estariam relacionados, tanto no controle da sua quiescência (inibição), quanto na indução da atividade citotóxica no ambiente uterino durante a gestação.

Verifica-se neste momento o rápido e crescente avanço nos conhecimentos acerca das células NK, os quais tem sido determinantes para caracterizar a população de células NK do ambiente uterino através das semelhanças com as células NKc. Porém, os estudos meramente comparativos entre as NKc em geral com as NKu, atingiram o seu nível limítrofe ao não permitir identificar o que estas últimas apresentam de peculiar e específico. Esta limita, por sua vez, as estratégias para elucidar e explicar a participação funcional das NKu na gestação. Desta forma, é notório que, um dos fatores limitantes neste avanço é a inexistência de um marcador celular específico para as células NKu de camundongos, assim como, a falta de um conjunto metodológico que permita isolar e purificar esta população celular, para o desenvolvimento de ensaios "in vitro" de maior precisão.

No contexto acima e com o intuito de contribuir para a compreensão da biologia das células NK presentes no ambiente uterino durante a gestação, o presente trabalho focalizou três conjuntos de experimentos, cada qual com estratégias definidas de acordo com os objetivos estabelecidos.

No primeiro, constante do capítulo II, intitulado "Subset classification of mouse uterine *Natural Killer* cells by DBA lectin reactivity" procurou-se identificar um marcador específico para as células NKu de camundongos.

Os relatos sobre a afinidade das células NKu de camundongos para com algumas lectinas (Damjanov e Damjanov, 1992; Stewart and Webstar, 1997) nortearam os trabalhos de Correa da Silva (1997), que demonstrou a lectina *Dolichos biflorus* (DBA) como sendo aquela seletiva para as células NKu. Baseado nestes dados, foram realizados experimentos para avaliar quão específica seria a reação lectina DBA-células NKu, para a identificação morfológica, localização e distribuição no ambiente uterino.

No capítulo III, intitulado "Functional Analysis of Murine Uterine Natural Killer Cells Genetically Devoid of Estrogen Receptors", procurou-se responder, de forma definitiva, se ocorre a ação direta do estrógeno sobre as células NKu de camundongos. Esta questão mereceu atenção, em se considerando que a migração das formas precursoras das NKu para o ambiente uterino ocorre exclusivamente na gestação em camundongos (Stewart and Peel, 1980; Peel, 1989; Zheng et al, 1991; Croy et al, 1997[b]) e ainda, pelo pressuposto de que o estrógeno poderia estar relacionado também com a proliferação e diferenciação das células NK no ambiente uterino (Stewart, 1987; Peel, 1989). Estudos utilizando RT- PCR e células de hibridoma, formado de células NKu e células SP2/0, demonstraram que estas células resultantes expressaram receptor de estrógeno α (ERα) (van den Heuvel et al., 1996). No entanto, Ogle e colaboradores (1997) relataram a expressão de receptores de estrógeno (ER) e receptores de progesterona (PR) na decidua basal dos sítios de implantação através de imunocitoquímica, porém, a reação positiva não foi observada nas células NKu. Os relatos existentes são portanto conflitantes, além de inconclusivos se considerado que, os receptores de estrógenos apresentam dois isotipos, os ERα e ERβ (Nilsson et al., 2001)

Para elucidar esta questão adotou-se a utilização de animais geneticamente modificados, com genes dos receptores de estrógeno α e β depletados (ERKO α ou β,) como doadores de transplantes de medula óssea para camundongos recipientes também geneticamente modificados, estes deficientes

em linfócitos. As análises morfológicas do útero reconstituído e o RT-PCR, realizado a partir de células isoladas pelo método lectina DBA-magnético, comprovam que o estrógeno não atua diretamente sobre as células NKu de camundongos.

No capítulo IV, intitulado "LY49 Gene Expression by Natural Killer Cells Localized to Mouse Uterus", procurou-se determinar a expressão temporal dos genes da família LY49 durante a gestação. Os receptores da família Ly-49 presentes nas NKc de camundongos são funcionalmente análogos aos KIR (*Killer imunoglobulin-like receptors*) de humanos (Brennan et al, 1996; Hiby et al, 1997; King et al, 2000; Raulet et al, 2001; Trowsdale, 2001), os quais são receptores do tipo imunoglobulina, envolvidos na regulação da citotoxicidade de NK. (Lanier, 1998; Raulet et al., 2001; Trowsdale, 2001). As Nku de humanos expressam um repertório completo de receptores KIR (Hiby et al., 1997; Verma et al., 1997), Camundongos C57Bl/6 (B6) possuem 11 genes funcionais e 5 pseudogenes para LY49, contidos no cromossomo 6 (Yokoyama e Seaman, 1993; Lanier, 1998; Smith et al, 1994; McQueen et al, 1998; Raulet et al, 2001; Makrigiannis et al, 2002). A ligação do MHC I ao Ly49A, C ou G inibe a citotoxicidade das células NK, enquanto se ligado ao LY49D ativa esta citotoxicidade (Mason et al, 1996; Makrigiannis et al, 2002). Os LY49D e H associam-se com uma molécula também envolvida na ativação das NK denominada DAP-12 (Mason et al, 1996; Smith et al, 1998). Esta família de genes LY49 tem, portanto, uma diversidade de ação nas NK, mas cuja expressão não fora ainda determinada para as células do ambiente uterino. Neste experimento, determinou-se a expressão de 14 genes da família LY49 e de DAP12 nos tecidos de diversas regiões do mesométrio por meio do RT-PCR, para avaliar as possíveis variações de expressão destes genes, de acordo com a região e período de gestação, estabelecendo as suas implicações na modelação funcional das NKu.

O conjunto destes experimentos, apresentados nos capítulos subsequentes, resultou cada qual

em artigos científicos submetidos para publicação. Por esta razão, cada capítulo apresenta formatação específica, de acordo com as normas editoriais das revistas.

2- OBJETIVOS

Os objetivos do presente trabalho foram:

- Caracterizar a lectina *Dolichos biflorus* (DBA) como potencial marcador seletivo para as células *Natural Killer* uterina (NKu) de camundongos e através deste marcador, restabelecer os parâmetros morfológicos de identificação, incidência e distribuição das NKu na gestação.

- Avaliar a expressão de receptores de estrógeno α e β através do RT-PCR em células NKu isoladas e a relação deste hormônio com migração, proliferação e diferenciação das células NK no ambiente uterino.

- Determinar o padrão de expressão dos genes da família LY49 nas células NK e em outros compartimentos do útero, comparados entre períodos de gestação diferentes e, entre camundongos normais e geneticamente modificados com deleção de populações linfocitárias.

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CAPÍTULO II

Subset classification of mouse uterine Natural Killer cells by DBA lectin reactivity

V.A. Paffaro, Jr ., M.C. Bizinotto , P.P. Joazeiro and A. T. Yamada

Laboratory of Cytochemistry and Immunocytochemistry, Department of Histology and Embryology, Institute of Biology, University of Campinas, 13083-970, Campinas, SP. Brazil

Correspondence should be addressed to:
Aureo T. Yamada
Department of Histology and Embryology
Institute of Biology, University of Campinas
Cidade Universitaria Zeferino Vaz
13083-970, Campinas, SP. Brazil

Phone: *55-19-37886252
Fax: *55-19-32570024
E-mail: yamadat@unicamp.br

Running head: Mouse uNK cell subsets.

ABSTRACT

Uterine Natural Killer (uNK) cells are a transient lymphocyte population found in the pregnant uteri of human and rodents. The pregnant uterine environment appears to influence migration, differentiation and suppression of the cytolytic activation of uNK cells but the mechanisms involved in these processes are not well understood. Similarities to circulating NK (cNK) cells are limited. The present study sought to discrimate uNK cells from cNK cells in mice by identification of a unique uNK cell marker. *Dolichos biflorus* (DBA) lectin, which has high selectivity for glycoconjugates containing N-acetyl D-galactosomine in the terminal position, reacted with the plasma membranes of mouse uNK cells. DBA lectin did not react with other uterine lymphocytes or with cNK cell surfaces in Swiss, CBA-J, C57BL/6, SJL, BALB/c, DBA-2 mice strains. DBA lectin staining was useful for both light and electron microscopy and distinguished 4 uNK cell subtypes that appear to be stages of differentiation. Quantitative evaluation of these 4 uNK cell subtypes over early to late gestational times showed dynamic changes between immature and mature forms in different compartments of the implantation sites and indicated the occurrence of microdomains in the uterus capable of controlling uNK cell proliferation and differentiation. This is the first report showing mouse uNK cells expressing specific molecules not found in other NK cells. Use of this reagent should enhance studies of earlier, non-granulated forms of uNK cells and provide new strategies for purification of mouse uNK cells for functional and molecular studies.

INTRODUCTION

In murine and human pregnancy are characteristics the influx of large numbers of NK lymphocytes in the uterus. These pregnancy-associated uterine NK cells (uNK) differentiate from progenitor cells derived from lympho-haematopoietic tissues outside of the uterus, particularly secondary lymphoid organs (Chantakru et al, 2002). In mice, a few, small, agranular lymphocytes appear around the 5th gestation day (gd) in the mesometrial region of each implantation site (Zheng et al, 1991; Croy et al, 1997^{b}). As gestation advances, these cells proliferate, differentiate and enlarge to become cells up to 50 μm in diameter. The enlarged cells have abundant cytoplasmic granules (Stewart and Peel, 1981; Head, 1996) and accumulate in mesometrial lymphoid aggregate of the pregnancy -MLAp (Croy, 1999) also called metrial gland (Wislocki et al, 1957; Stewart and Peel, 1980). UNK cells are transient because they are not seen in the uterus after parturition (Stewart and Peel, 1980; Peel, 1989; Delgado et al, 1996).

Immunophenotyping studies in both rodents and humans indicate that these pregnancy-associated transient lymphocytes resemble some peripheral NK cell subsets (Bernard et al, 1978; Mukhtar, et al, 1989; Readline and Lu, 1989; Linnemeyer and Hamilton, 1990; King and Loke, 1991; Croy and Kiso, 1993; Head, 1996; Loke and King, 2000). The unequivocal identification of these cells as NK lineage was demonstrated in a very elegant series of experiments using several strains of genetically-modified, lymphocyte deficient mice and transplantation of cells collected from primary and secondary haemopoietic organs (Croy and Kiso, 1993; Guimond et al, 1997; Croy et al, 2000; Greenwood et al, 2000; Chantakru et al, 2002,). Human uterine CD56+, CD16^{-}, CD3^{-} NK cells appear to be analogous to mouse uNK cells, and represent >70% of the leukocytes found in late secretory phase of endometrium and in early gestational decidua (Loke and King, 2000). These cells express a full repertoire of KIR (Killer imunoglobulin-like

receptors) (Hiby et al, 1997), which are Ig-like receptors involved in the regulation of NK cell cytotoxicity (Lanier, 1998; Raulet, Vance and McMahon, 2001; Trowsdale, 2001). The LY-49 family receptors in mice are functionally analogous to human KIR (Brennan et al, 1996; King et al, 2000; Raulet et al, 2001; Trowsdale, 2001) but thorough analysis of these receptors is not yet reported on mouse uNK cells.

The function of uNK cells in the pregnant mouse uterus has been suggested using knockout mice. UNK cells are responsible for Interferon-γ (IFN-γ) production (Ashkar and Croy, 1999) and the absence of either uNK cells or IFN-γ, prevents the normal, pregnancy-induced changes in endometrial blood vessels known as spiral arteries, and reduces decidual integrity. Neither uNK cells nor IFN-γ are required for fetal survival to term (Ashkar and Croy, 1999; Ashkar et al, 2000).

The relationship of the uNK cell to other cells involved in innate immune responses is an intriguing question since, in uNK cells, cytolytic activity is not triggered with IFN-γ production (Stewart and Peel, 1993). A persisting difficulty in studying uNK cells is the absence of specific cell markers to readily distinguish uNK from other NK cells. Such markers could provide methods for isolation and purification of this cell population from the mouse uterus. Here we report the *Dolichos biflorus* (DBA) lectin is a very selective and sensitive reagent for discriminating mouse uNK cells from all other lymphocytes.

MATERIAL AND METHODS

Animals

Randombred (Swiss) and inbred (CBA-J, C57BL/6, SJL, BALB/c, DBA-2) female mice were purchased from Animal Laboratory facility of University of Campinas (CEMIB/UNICAMP) and mated with males of the same strain. The morning of vaginal plug detection was considered gestation day (gd) one. Virgin or pregnant mice on desired gd were sacrificed by perfusion with fixative solution after deep anesthesia with ketamine (Virbac, Brazil) and xylazine chloridrate (Virbac, Brazil). All animal handling and euthanasia were conducted under approved protocols.

Tissue Processing for Light Microscopy

To collect tissues for light microscopy virgin and pregnant (5^{th} to 17^{th} gd) mice were perfused through the left ventricle with 30mL of 4% paraformaldehyde (Sigma, St. Louis, USA) in 50 mM phosphate buffer-saline (PBS) fixative solution. The uteri from both pregnant and virgin mice of all strains were dissected and processed for conventional paraffin embedding. In the same way, the thymus, spleen and lymph nodes of virgin Swiss and C57BL/6 mice were collected and processed for paraffin embedding.

Paraffin embedded uterus of pregnant Rag-$2^{-/-}/\gamma c^{-/-}$ double knock-out mice (B^-, T^-, and NK^- deficient) and Rag-$2^{-/-}/\gamma c^{-/-}$ mice transplanted with bone marrow cells from ICR *scid/scid* (B^-, T^-, and NK^+) or C57BL/6 mice were kindly donated by Dr. Anne Croy (University of Guelph, Canada).

Tissue Processing for Electron Microscopy

To collect material for ultrastructural analysis, pregnant mice (8^{th} and 10^{th} gd) were perfused with 30mL fixative solution comprised of 2% paraformaldehyde, 1.5% glutaraldehyde (EM Science, USA) and 0.1M sucrose in 100 mM PBS, pH 7.4. Implantation sites were dissected, washed in PBS, post fixed with 1% OsO_4 and further processed for conventional epoxy resin (Epon, PolyScience Inc, USA) embedding. Ultrathin sections were collected on copper grids, stained with uranyl acetate and lead citrate (Fluka, Switzerland) and examined with LEO EM906 transmission electron microscope at 80kVA.

For ultrastructural cytochemistry, pregnant mice (8 and 10 gd) were perfusion fixed with 30mL of 4% paraformaldehyde, 0.2% glutaraldehyde and 0.1M sucrose in 100 mM PBS, pH 7.4 solution. After washing with 50 mM PBS, the samples were dehydrated with N'N'-dimethylformamide and embedded with LR-White resin (Sigma, St Louis, USA) at –20°C and UV polymerization in a freeze-substitution unit (FSU10, BAL-TEC, Swiss). Ultrathin sections were collected on nickel grids for cytochemistry.

DBA Lectin Cytochemistry

Light Microscopy: Paraffin sections of virgin and pregnant uteri of randonbred Swiss (5^{th} to 17^{th} gd), and inbred CBA-J, C57BL/6, SJL, BALB/c, DBA-2 mice (8^{th} or 10^{th} gd), Rag-$2^{-/-}/\gamma c^{-/-}$ and Rag-$2^{-/-}/\gamma c^{-/-}$ mice transplanted with *scid/scid* (10^{th} gd), and lymphoid organs (spleen, thymus and lymph nodes) of virgin Swiss and C57BL/6 mice were used. Deparaffinized sections were re-hydrated and treated with 0.3% hydrogen peroxide for 30 min. After washing with 50 mM PBS, the sections were incubated with 1% bovine serum albumin (BSA) in 100 mM PBS for 30 min, followed by biotinylated-*Dolichos biflorus* (DBA) lectin (Sigma, St. Louis,

USA), diluted 1:150 in 1%BSA-PBS, incubation overnight at 4°C. The PBS washed sections were reacted with streptoavidin-peroxidase (Chemicon, USA) for 30 min at room temperature and revealed with 3,3-diaminobenzidine (Sigma, St. Louis, USA) in 50 mM TBS containing 0.1% hydrogen peroxide. The sections were counter-stained with Harris' hematoxylin and mounted with Entellan (Merck, Germany). Alternatively, it was used FITC-conjugated DBA lectin (Sigma, St. Louis, USA) for fluorescence microscopy on paraffin sections. After re-hydration and blocking with 1% BSA/PBS for 30min, the sections were incubated with DBA-FITC, diluted 1:100 in 1% BSA/PBS, for 60min at room temperature, washed in ultrapure water and mounted with anti-fading medium (Vectashield, Vector Co, USA). DBA lectin specificity controls was performed by adding, 0.1 M N-acetyl, D-galactosamine (GalNac, Sigma, St. Louis, USA) into the DBA lectin solution or omitted the step of DBA lectin incubation.

Electron Microscopy: LR-White embedded ultrathin sections collected on nickel grids were quickly washed with 50 mM PBS, treated with 1% BSA-PBS for 30 min. and then incubated with biotinylated-DBA lectin (Sigma, St Louis, USA) diluted 1:300 in 1% BSA-PBS overnight in a humidity chamber at 4°C. After washing in 50 mM PBS, grids were incubated with streptavidin-gold 5 nm (Amersham, UK) for 30 min, washed with ultrapure water and uranyl-lead stained. Specificity control was 0.1 M GalNac mixed with the DBA lectin solution prior to its use.

Periodic Acid Shiff (PAS) Histochemistry

Paraffin sections of Swiss and C57BL/6 pregnant mice uteri (8^{th} and 10^{th} gd) were used for PAS staining after amylase treatment. The deparaffinized and re-hydrated sections were treated with 0.1% α-amylase (Sigma, St. Louis, USA), 30 min. at room temperature, washed and than

processed for conventional periodic acid-Shiff staining, counterstained with hematoxylin and mounted with Entellan.

Proliferating Cell Nuclear Antigen (PCNA) Immunocytochemistry

Paraffin sections of uteri from Swiss and C57BL/6 mice (7^{th} to 12^{th} gd) were used for PCNA immunostaining to detect dividing cells. Deparaffinized sections were incubated with rabbit polyclonal anti-PCNA (Chemicon, USA) followed by TRITC-conjugated goat anti-rabbit IgG (Vector, USA). After washing, the sections were stained with FITC-conjugated DBA lectin (Sigma, St. Louis, USA) and mounted with anti-fading medium (Vectashield, Vector Co., USA). The analyses were performed with fluorescence (λ=520 and 560 nm) and differential interference contrast (DIC) microscopy. Images were recovered with digital imaging system (CoolSnap, Media Cybernetics, USA).

Morphometry and Stereology

Samples of sections stained with DBA lectin/streptavidin-peroxidase were used for morphometry and stereological distribution analyses of DBA-reactive cells in the pregnant uterus (5^{th} to 17^{th} gd). For stereological evaluation, tree sections from 3 different implantation sites of 3 different animals for each gestational day were random chosen. The DBA lectin stained uNK cell subtypes were enumerated in a constant test area (TA=$21.8x10^4$ μm^2) by setting an eyepiece with 1mm2 graticule and 50X objective in a bright field microscope. Quantitative data were submitted to SAS computer statistic evaluation by Duncan's multiple range test for density. Significance was

considered at $p<0.01$. DBA lectin-positive cells in the placenta and within blood vessels were not included in the counts. To estimate the size of DBA lectin-positive cells, a screw micrometer eyepiece (ZEISS, Germany) and 100X objective previously calibrated with standardized scale were used. Diameters of at least 50 cells from each four morphological categories of DBA lectin-positive cells were measured in 10 gd samples.

RESULTS

Characteristics of DBA Lectin-Reactive Cells by Light Microscopy

DBA (*Dolichos biflorus*) lectin cytochemistry performed on sections of pregnant mouse uterus showed a selective reaction pattern on cells localized restrictedly at mesometrial side (Fig.1a) at all gestation days, not found in the virgin uterus. The DBA positive reactions were localized by light microscopy to cell surfaces and to the cytoplasmic granules (Fig. 1b, 1c), which were completely inhibited by GalNac (Fig. 1d). DBA lectin reactivity occurred in the same cells that were in adjacent sections amylase resistant PAS-positive granules (Fig. 1e). These PAS-positive cells were formerly known as granulated metrial gland cells (GMG) and currently, as uterine NK cells. The DBA lectin-positive cells were restricted to mesometrial side of each implantation site including the mesometrial lymphoid aggregate of pregnancy (MLAp) and were found in all immune-competent mice strains tested (Swiss, CBA-J, C57BL/6, SJL, BALB/c, DBA-2). However, no DBA-positive reaction was seen on cells of thymus, spleen or lymph node (Figs. 1f, 1g). These evidences suggested the high selectivity of DBA lectin to recognize uNK cells.

The $Rag^{-/-}/\gamma c^{-/-}$ mice, which are alymphoid, had no DBA lectin-positive cells in the pregnant uterus (Fig. 1h), but the positive cells were seen in those $Rag^{-/-}/\gamma c^{-/-}$ recipient mice

restored with bone marrow from SCID donors or BM/lymph node cells from C57Bl/6 wild type donors (Fig. 1i), confirming the affinity and specificity of DBA lectin to mouse uNK cells.

Reaction was occasionally seen on endothelial cells of some capillaries and small veins (data not showed) present in non-decidualized endometrium, and on endothelium of large veins in the myometrial and mesometrium (broad ligament) both in virgin and pregnant uterus.

Four morphological subtypes of uNK cells always DBA lectin-positive, with heterogeneous chromatin compactness and cytoplasmic granules contents, were found in the pregnant uterus. Their distinct features correlated to their size (diameter). The subtype I cells were round, small lymphocyte-like cells (9 ±3 μm) without granules. These cells had a round nucleus and were strongly DBA lectin-positive at the cell surface (Fig. 2a). The subtype II cells were slightly larger (13±2 μm), DBA-positive at cell surface and had a few DBA- positive cytoplasmic granules (Fig. 2b). Subtype III cells were much larger (26±7 μm), with round euchromatic nuclei, heavily granulated and still maintained a round-shape. A strong DBA lectin-positive reaction was found on both the granules and surface of subtype III cells (Figs. 1b, 1c, 2c). Subtype IV cells were the DBA lectin-positive largest granulated cells (30±4 μm). Many had abnormally condensed chromatin (apoptotic-like) in the nucleus and the DBA lectin-positive granules were distributed in the vacuolated-like cytoplasm (Fig. 2d). These subtypes were not randomly distributed in the uNK cell enriched region through the gestation days.

Ultrastructural Features of DBA Lectin Reactive Cells

The four subtypes of DBA-positive uNK cells were also distinguished ultrastructurally. Subtype I cells were small round cells with scattered cytoplasm containing few membranous organelles

restricted to rough endoplasmic reticulum and nucleus with large clumps of condensed chromatin. No granule or Golgi complex cisternae were seen (fig 3a).

Subtype II DBA-reactive uNK cells were round-shaped cells with a round nucleus that still contained clumps of condensed chromatin. The rough endoplasmic reticulum (RER) cisternae were randomly distributed and Golgi complex cisternae were seen assembled nearly to centrosomes (Fig. 3b). A few secretory lysosome type granules (Fig. 3f) were found in the cytoplasm around the Golgi cisternae. Microvilli and phylopodia-like projections were often seen at the cell surfaces.

Subtype III DBA lectin positive uNK cells were large cells with indented nuclei. The nuclei were packed with euchromatin and conspicuous nucleoli (Fig. 3c). Typical secretory lysosome granules with homogeneous central core and peripheral electron dense areas containing densely packed microvesicles were distributed preferentially near the well-organized Golgi complex cisternae (Fig. 3c, 3e, 3f). The size, shape and compactness of the granules varied but a common feature was their double compartmentalization with a peripheral electron dense cap containing microvesicles and a homogeneous core. The RER were widely distributed in the glycogen rich cytoplasm. Pseudopodia-like and large cell projections were seen in close contact with stromal or endothelial cells (Fig. 3c), but no membrane mediated cell junctions were seen between uNK cells or other cell types.

Subtype IV DBA- positive uNK cells were characterized as large and irregularly shaped cells with numerous granules, resembling those in subtype III cells, distributed in the cytoplasm. The cytoplasm usually showed large electron lucent areas, probably due to glycogen extraction. The RER were packed in a parallel array and their contents were more electrondense. Golgi complex cisternae were less evident and the round nucleus showed thin clumps of chromatin in an apoptotic-like profile (Fig. 3d). The DBA lectin-gold both labeled the membranous caps and

electrondense core contents of granules, as well as, the cell membrane at its extra cellular surface (Figs. 3g, 3h). Thus, it is a useful reagent for study uNK cells at ultrastructural level.

Distribution and Quantification of DBA Lectin + uNK Cells

DBA-positive uNK cells were not found in the uterus of virgin mice or until 4^{th} gd. After 5^{th} gd, DBA- positive uNK cells were seen exclusively in the mesometrial side of each implantation site. Due to the continuous anatomical modification of the uterus during gestational, four arbitrary anatomical regions were defined to quantify the distribution of uNK cell subtypes within the mesometrial tissue (Fig. 4). Distribution of each cell subtypes within each region between 5^{th} to 17^{th} gd is shown in Figure 5.

Subtype I uNK cells were first found in the region 2 on 5^{th} gd (2/TA) and than in the region 1 on 6^{th} gd (9/TA). Here, they increased gradually in number reaching their peak on 10^{th} gd (67/TA). In region 2, the highest numbers of subtype I cells were observed on 8^{th} gd (32/TA) and then declined gradually. In regions 3 and 4 this subtype was rarely found only on 8^{th} gd.

Subtype II uNK cells were first seen in region 2 on 5^{th} gd (1.8/TA). Their numbers peaked in this region on 8^{th} gd (170/TA), and they decreased, disappearing completely at 12^{th} gd. In region 1, subtype II was found after 6^{th} gd (12/TA), and numbers increased on 9^{th} to 10^{th} gd (107/TA) and suddenly decreased on 12^{th} gd (2/TA). In regions 3 and 4, a few subtype II cells were observed by 6^{th} gd. They increased progressively until 9^{th} gd (27/TA), but in a quarter amount if compared to region 1. Then, this subtype decreased gradually and did not disappear from these regions until 17^{th} gd.

Subtype III uNK cells were only found after 6^{th} gd in regions 2, 3 and 4. The greatest numbers of this subtype were seen in region 3 on 9^{th} gd (100/TA) with a significant reduction in

the number of these cells on 10^{th} gd (48/TA) and a second major drop on 15^{th} gd (4/TA). In region I a significant number of this subtype was found after 10^{th} (24/TA) and kept until17^{th}.

Subtype IV (apoptotic) uNK cells were consistently found after 8^{th} gd in the regions 2 (3/TA), 3 (1.5/TA) and 4 (2.2/TA) and become the most representative form of uNK cells found in the uterus after 12^{th} gd. The first dramatic gain in the numbers of this subtype occurred on 9^{th} gd (68/TA) in region 3 and continued on 10^{th} gd (79/TA), but while it gradually decreased after12^{th} gd in this region, the peak number was seen moved to the region 2 (86/TA). Then, its numbers declined until gd 17^{th}gd in all regions considered.

PCNA Immunocytochemistry

High amount of PCNA-positive nuclei were seen on pre-decidual and decidualized cells localized to the antimesometrial side (Figs.2e, 2f) and in the nuclei of uNK cells at mesometrial side (Fig. 2g). Double labeling performed with anti-PCNA and DBA lectin identified subtype II uNK cells as having the highest proliferative activity (Fig. 2g). Subtype IV cells did not stain with PCNA. The greatest number of PCNA labeled cells was concentrated in region 2 from 7^{th} to 10^{th} gd. Only a few of the dually stained uNK cells were localized in region 3 after 8^{th} gd. No PCNA labeling was distinguishable in DBA lectin-positive uUK cells after 15^{th} gd.

DISCUSSION

Lectin cytochemical characterization of the pregnant mouse uterus had previously identified uNK cells, formerly called granulated metrial gland (GMG) cells, as cells positively labeled by

Dolichos biflorus agglutinin (DBA) lectin and other lectins (Damjanov and Danjanov, 1992; Stewart and Webster, 1997). Screening of 16 lectins performed in our laboratory, with differing cytochemical methods, found several types of labeled cells in paraffin or cryosections of virgin and pregnant mice uteri (unpublished data). However, DBA lectin was the only lectin reacted with glycoconjugates exclusively expressed by uNK cells and due to its high affinity for a specific sugar, is as suitable as antibody reagents for phenotypic identification of cells. DBA lectin appears to be a broadly useful reagent because it reacts with uNK cells on all gestational days, at all stages of uNK cell differentiation and in all mice strains of normal mice studied to date. Positive reactions localized on both cell surface and granule contents were completely inhibited by GalNac, the specific inhibitory sugar of DBA lectin (Wu and Sugii, 1988), which characterized the acquisition of glycoconjugates containing GalNac as a lineage differentiation step shown only by NK cells reaching the pregnant uterus.

PAS staining has been more widely used to identify uNK cells in the rodents (Bulmer, 1968; Croy et al, 1997[a]). The glycoconjugate contents in rodent uNK cell granules react strongly with Shiff' reagent after periodate treatment. However, DBA lectin, as used in the protocol reported here, was more sensitive than PAS for identification and localization of mouse uNK cells. First, DBA lectin reacts against glycoconjugates present not only in the granules but also at the uNK cell surface. This was demonstrated both by light and electron microscopy and permits investigators to confidently identify the immature form of uNK cells that are PAS negative. The diameter of agranular DBA lectin-positive uterine lymphocytes is smaller than has been reported for uNK cells by others (Bernard, 1978; Delgado et al, 1996). Interestingly, DBA lectin reacts with granules in IL-2 activated splenic NK cells obtained in culture, but the plasma membranes of these cells are not reactive (Fonseca, 2000). This means that DBA lectin can be used for mice

to distinguish uNK cells from activated circulating NK cells in a manner analogous to the use of CD56 as a marker for human uNK cells (King and Loke, 1991; Hiby et al, 1997).

Second, the correlative image analysis between DBA lectin-labeled uNK cells by light microscopy and ultrastructure, characterized four morphologically distinguishable maturation stages of uNK cells. The ultrastructure of uNK cells reported by Peel (1989) and by Parr et al (1990) and others are coincident with our observations of DBA lectin-positive lymphocytes. We confirmed the presence of an immature form of uNK cells without granules resembling the morphology of small lymphocytes, but DBA lectin cytochemistry recognized the glycoconjugates on their plasma membrane. These are probably progenitor immature cells that migrate to the uterus preferentially from secondary lymphoid organs (Chantakru et al, 2002) and then differentiate to the intermediate uNK cell subtype characterized as subtype II.

Our study identifies the first day of progenitor recruitment as gd 5 and identified the microdomain initially colonized by uNK cells. Further, it suggests that the recruited cells are already committed to the uNK cell lineage (DBA-positive) but are not yet activated to terminally differentiate [i.e. enlarge, divide (PCNA-negative) or acquire granules (agranular)]. The significance of this observation is that triggering of activation of uNK cells can now be addressed as an intra-uterine event, and not an event initiated by interactions with endothelial cells as the uNK cell precursors move into uterine tissue. The exquisite microdomain localization of immature uNK cells is coincident with work of Kruse et al (2002) showing that endothelial cell addressing vary within the mesometrial tissue of pregnant uteri. Since, decidua basalis has a unique "address" of VCAM-1 only, it strongly suggests this could be the recruitment signal.

The most evident morphological feature of differentiation of uNK cells was the gradual building of membranous organelles in the cytoplasm, especially the Golgi complex concentrated around the centrosome and the granules with double compartments. These typical double

compartment granules, known as secretory lysosomes (Griffiths, 1995; Stinchcombe and Griffiths, 1999), are useful for uNK cell identification by electron microscopy. UNK cells have been considered fully mature or differentiated, once their granules contain the proteins related to cytolytic activity, such as perforin, granzymes and lysosomal enzymes (Pell, 1989; Parr et al, 1990; Zheng et al, 1991; Allen and Nilsen-Hamilton, 1998), which would be cells classified as subtype III by our DBA lectin cytochemistry and TEM criteria.

Senescence of uNK cells is well known but our studies highlight how early it begins (gd 8), implying the life span of an individual uNK cell may be as short as 3-4 days. The onset of death in the uNK cell population also supports previous studies suggesting that the major functions of uNK cells have been accomplished by mid-gestation. These functions appear to include support of decidual cells from gd 7 and modification of maternal spiral arteries by gd 11 (Greenwood et al, 2000, Ashkar et al, 2000). Cells of subtype IV could be considered as those committed to senescence not apoptosis, because the cells are not rapidly cleared from the tissue. Delgado et al (1996) reported that uNK cells committed to programmed cell death, after 12^{th} gd.

The distribution analysis of the dividing, mature and senescent uNK cells also provides important new information. During mid-pregnancy (9^{th}-11^{th} gd) regions closer to the placenta show increases in mature forms (subtypes III and IV). In contrast increases in immature and proliferative stages are concentrated near or within the myometrium. The differences in the distribution of the uNK cell subtypes suggest gradients signaling uNK cell maturation within the pregnant uterus. Gradients could be established by myometrial, decidual and/or embryo-derived factors. Very few uNK cells belonging to the subtypes I and II are found in regions 3 and 4 during gestation. Interesting was the high incident of PCNA-positive subtype II and III cells in region 2. Although the number of subtype I and II cells decrease at and after 9^{th} gd in this region, there is no corresponding increase of subtypes III or IV in subsequent gd in the same location.

This suggests the more mature subtypes III and IV are terminally differentiated, post-mitotic migrating cells recruited towards the placenta. This also suggest proliferative activity is highly regulated by unknown influences and that uNK precursor cells function declines from 9th gd and is totally lost after the 12th gd, since after this time-point almost all uNK cells are mature type III or senescent type IV cells. Although PCNA-DBA lectin dual positive cells were detected until 12th gd there were no corresponding increases in uNK cells.

This study has shown that uNK cells express specific glycoconjugates containing GalNac on their surface. Investigations are in progress to isolate and characterize these glycoconjugates. It is also the first report to fully characterize where the uNK cell population specifically resides after migration to the uterus, where cell division occurs and to demonstrate attraction of only post-mitotic uNK cells towards the placenta.

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ACKNOWLEDGEMENTS

This work was supported by grants from FAPESP, CNPq and FAEP/UNICAMP to ATY. V.A.P.Jr received a scholarship from CAPES and FAPESP. M.C.B received a scholarship from CNPq. We are grateful to Dr. Anne Croy for reviewing the manuscript and for valuable suggestions in discussion of results.

FIGURE LEGENDS

Figure 1. Photomicrographs of mouse pregnant uterus. (a) Low magnification of cross-sectioned uterus (Swiss) on 8th gd showing distribution of DBA-reactive cells (brown areas pointed out by arrows) only on the mesometrial (M) side, (E - embryo). (b) DBA lectin reaction pattern on typical uNK cells on 8th gd (Swiss) showing a well-labeled cell surface (arrow heads) and labeled cytoplasmic granules, streptoavidin-diaminobenzidine reaction. (c) FITC-DBA lectin reaction on surface and granules of uNK cells on 10th gd (C57BL/6). (d) GalNac inhibited DBA lectin cytochemistry shows no reaction with uNK cells (arrows) on 8th gd (C57BL/6). (e) PAS staining showing positive reactions in the granules (arrowhead) of uNK cells on 8th gd (Swiss). (f, g) no DBA-positive reaction is observed either in the thymus nor spleen cells of virgin C57BL/6 mice, respectively. (h) pregnant uterus of alymphoid Rag2$^{-/-}$/γc$^{-/-}$ mice lacks DBA positive cells. (i) pregnant Rag2$^{-/-}$/γc$^{-/-}$ uterus gained DBA-positive uNK cells after the mouse was transplanted with bone marrow cells from a C57BL/6 donor.

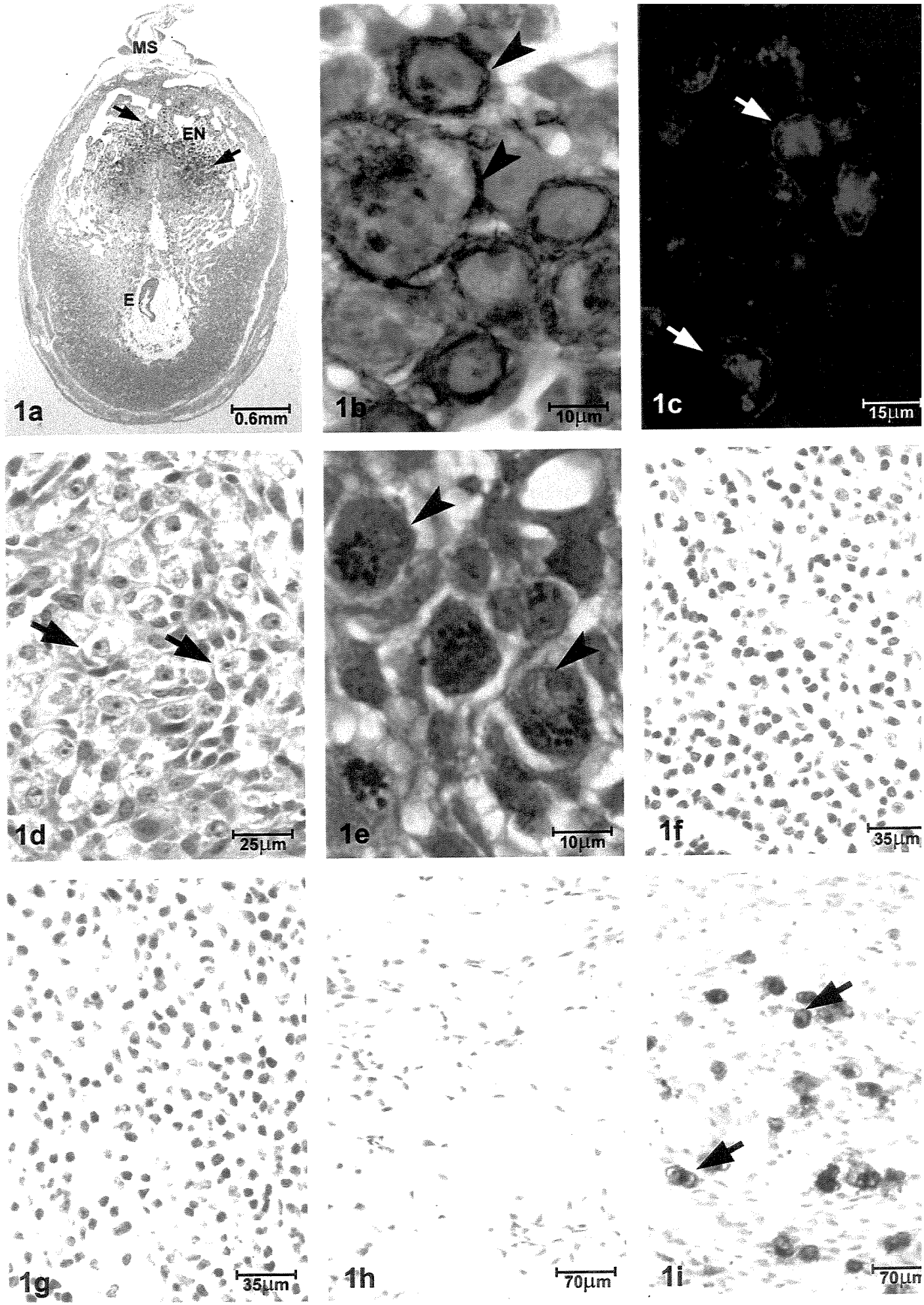
Figure 2. DBA lectin staining pattern after peroxidase/diaminobenzidine distinguished mouse uNK cells subtypes I (a), II (b), III (c), IV (d). e) anti-PCNA-positive nuclei (red) of decidualized cells at anti-mesometrial side of Swiss mice uterus on 9th gd and corresponding DIC image in (f). g) anti-PCNA and DBA lectin double labeling showing PCNA-positive nuclei (red) and uNK cells (green) present in MLAp.

Figure 3. Electron micrographs showing ultrastructure of uNK cells subtypes.
(a) subtype I is likely a small lymphocyte without granules and nucleus (N) with large heterochromatin clumps (N). (b) subtype II, showing a few granules (*) and cisternae of rough endoplasmic reticulum (RER) and Golgi complex (GC). (c) subtype III, the mature form of uNK cells with nucleus with few heterochromatin clumps and cytoplasm containing large numbers of granules (*) and well-organized Golgi complex (GC) and rough endoplasmic reticulum (RER). (d) subtype IV, showing large electronlucente area in the cytoplasm with stacked cisternae of RER and the nucleus (N). (e) detail of subtype III uNK cell cytoplasm showing accumulation of granules (*) around the centrosome area with centrioles (c) and Golgi complex (GC). (f) detail of a typical secretory lysosome granule showing its double compartmentalization with a peripheral

electron dense cap containing microvesicles and the homogeneous core. (g, h) DBA lectin ultrastructural cytochemistry, showing gold particles distributed on the extracellular face of plasma membrane (arrow heads) and in the granules (*).

Figure 4. Line diagrams representing uterus on 5^{th} (A), 10^{th} (B), and 15^{th} (C) gestational days. The squares represents the regions used to quantify uNK cells subtypes distributed in the mesometrial side (M). The opposite antimesometrial side (AM) corresponds to the embryo implantation site. Region 1 (R1) matches the myometrium (Mi) around the mesometrium and endometrium (En), where the mesometrial lymphoid aggregate of the pregnancy (MLAp) develops by 8^{th}-10^{th} gd. Region 2 (R2) is the intermediate endometrial region between R1 and R3 where, until 8^{th} gd, the uterine luminal epithelium (LE) persists. Region 3 (R3) is the major decidualized endometrium nearly localized to the developing conceptus (E) until 8^{th} gd and after this, (from 9^{th} to17^{th} gd), is the region of maternal interface closely apposed to the foetal placenta (FP). Region 4 (R4) is the decidualized endometrium lateral to R3. (GTC)- giant trophoblast cells layer.

Figure 5. Distribution of uNK subtypes I (●), II (△), III (■) and IV (○) cells per Test Area (TA = $21{,}8x10^4$ μm^2) in the mesometrial regions 1 (R1), 2(R2), 3 (R3) and 4 (R4) of pregnant uterus from 5^{th} to 17^{th} gestational days (gd).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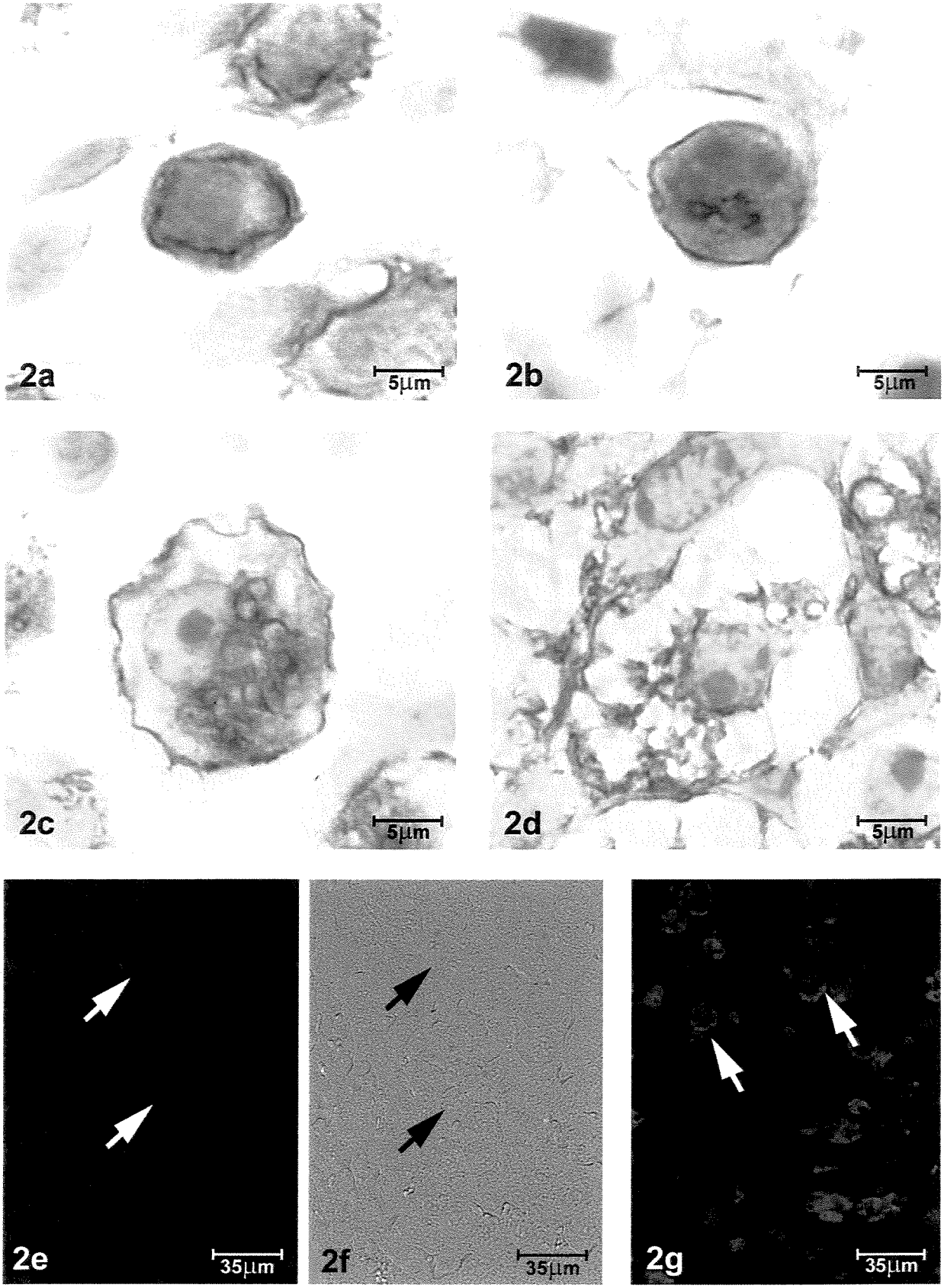
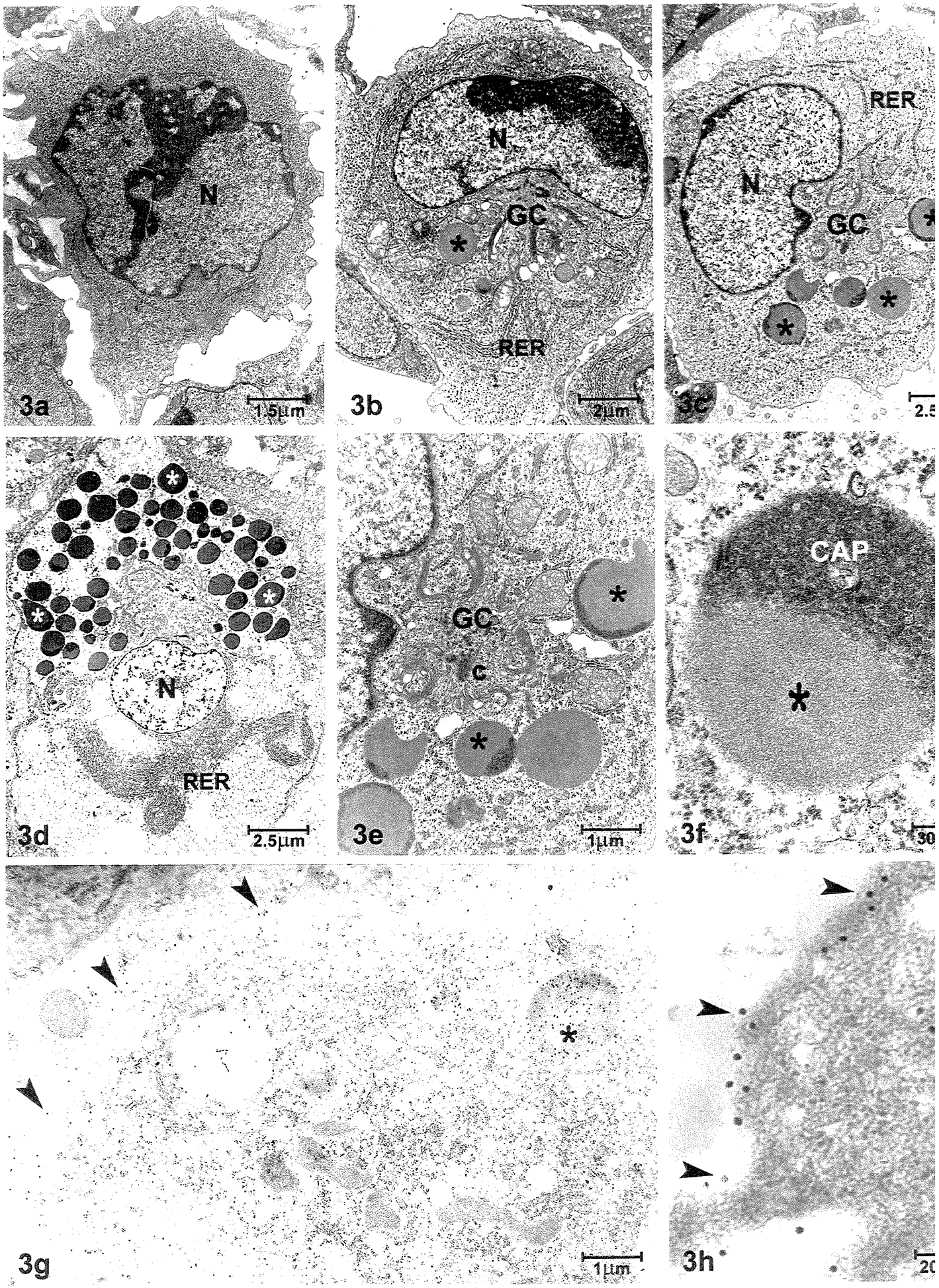


Figure 2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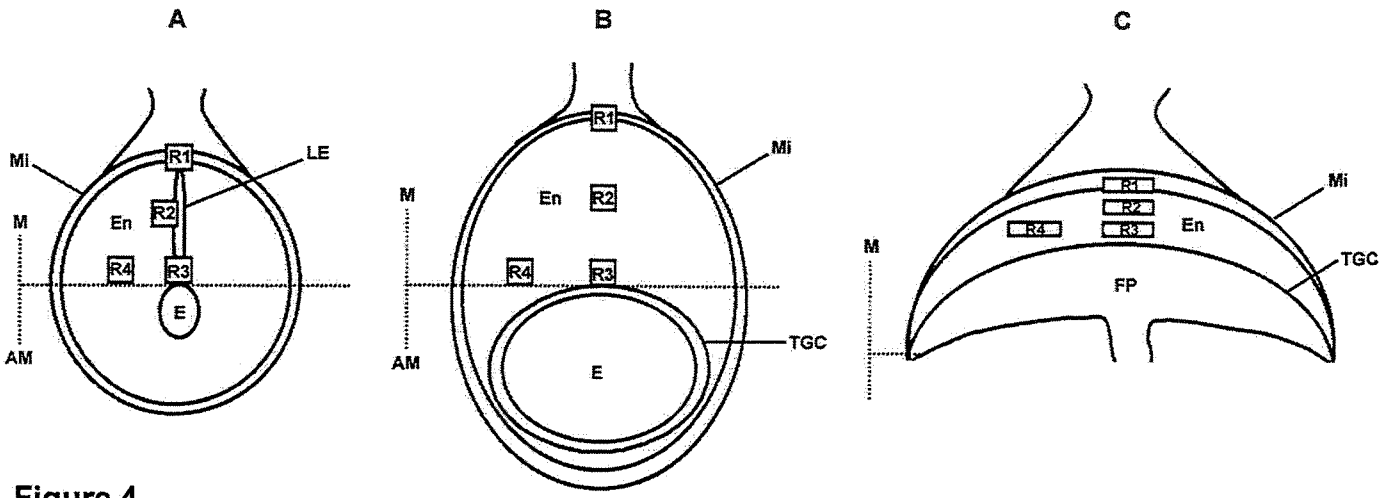

Figure 4

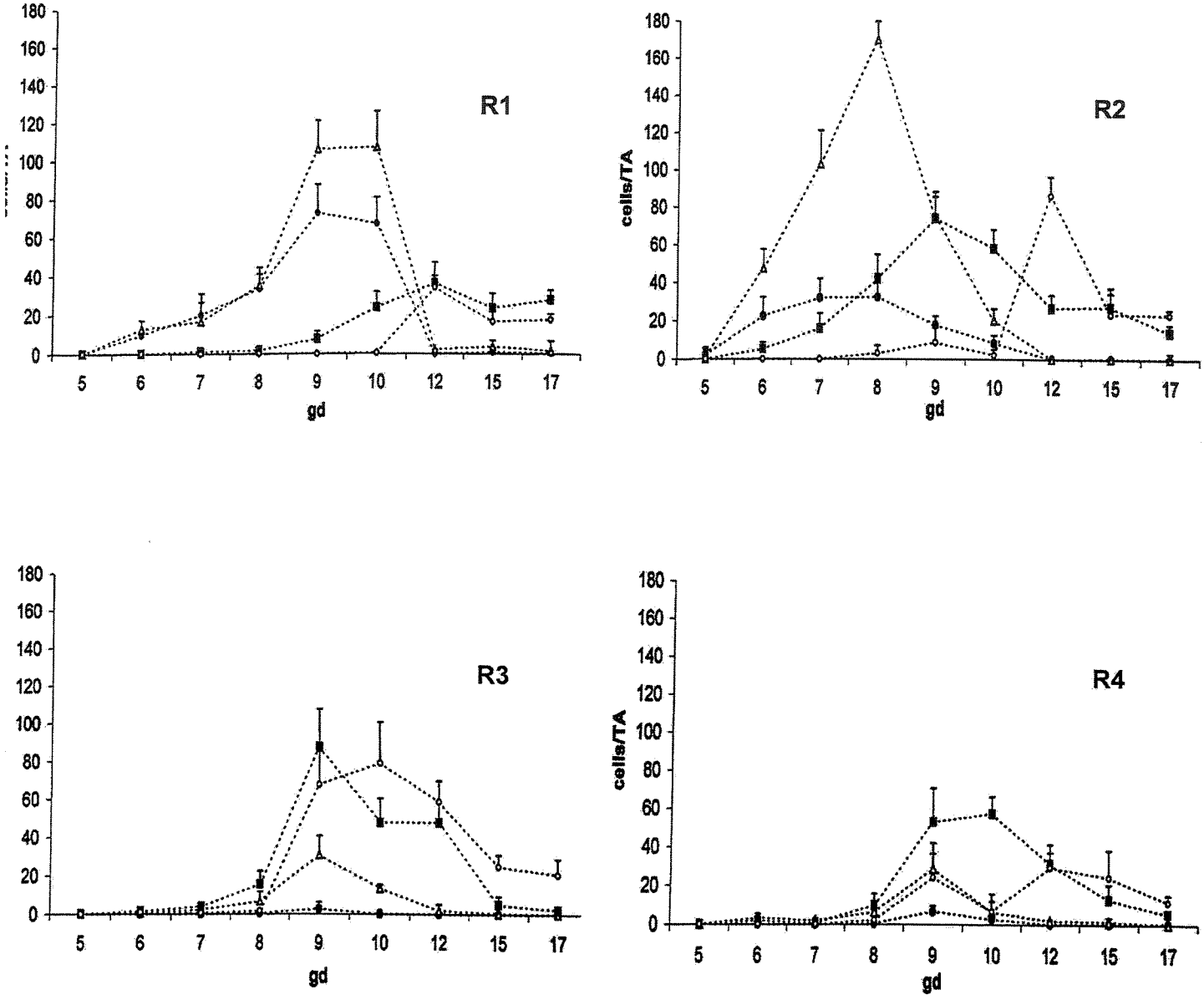
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Figure 5

CAPÍTULO-III

Functional Analysis of Murine Uterine Natural Killer Cells Genetically Devoid of Estrogen Receptors

A.M. Borzychowski[a], S. Chantakru[a], K. Minhas[a], V.A. Paffaro, Jr.[a, b], A.T. Yamada[b], H. He[a], K.S. Korach[c] and B.A. Croy[a, d]

[a] Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada, N1G 2W1, [b] Institute for Biology, UNICAMP, Campinas, SP Brazil 13083-970 and [c] Receptor Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institute of Health, P.O. Box 12233, Research Triangle Park, NC 27709

Running head: Uterine Natural Killer Cells

[d]To whom correspondence should be addressed at: Department of Biomedical Sciences, Room 2604, Building 40, University of Guelph, Guelph, Ontario, N1G 2W1, Canada. Phone: +1 (519) 824-4120, x4915; Fax: +1 (519) 767-1450; E-mail: acroy@uoguelph.ca

Abstract

Uterine Natural Killer (uNK) cell differentiation in vivo requires estrogen (E) priming prior to progesterone (P). Hybridomas between uNK precursor and SP2/0 cells express message for E receptor (ER)α but nor PR. However, mature, rodent and human uNK cells lack these receptors. To functionally assess requirements for uNK cell expression of ERα or ERβ during precursor differentiation, marrow was transplanted from either $ER\alpha^{o/o}$ (αERKO) or $ER\beta^{o/o}$ (βERKO) mice into alymphoid RAG-$2^{o/o}/\gamma c^{o/o}$ females. Recipients were mated and their implantation sites were examined by light microscopy, morphometry and ultrastructure. High numbers of uNK cells were established from each donor strain. Graft-derived uNK cells were similar in number and morphology to uNK cells of normal mice, suggesting that neither α- nor β-ER is required for uNK precursor cell differentiation. Induction of spiral artery modification in the transplant recipients indicated that graft-derived uNK cells had functional properties. A novel technique for rapid isolation of highly purified uNK cells from normal mice using Dolichos biflorus agglutinin (DBA) lectin-conjugated magnetic beads was employed to obtain RNA. Expression of α- and β-ER was absent by RT-PCR from NK cells isolated from the uterus, supporting the conclusions from the in vivo study.

Keywords: mouse pregnancy, lymphocyte differentiation, lymphocyte homing, decidual spiral artery modification

INTRODUCTION

Uterine natural killer (uNK) cells are the dominant endometrial lymphocytes of early gestation in many mammals, including women (Peel, 1989; Stewart. 1998; King, 2000). These cells depend on both estrogen and progesterone to promote their differentiation into large granulated cells that are absent from virgin or postpartum uteri (Stewart, 1987; Peel & Stewart, 1988). This life history pattern suggests that uNK cells have important, pregnancy-associated functions (Guimond, Wang and Croy, 1998). In mice, uNK cells can be recognized by gestation day (gd) 5 as rare, small, non-granulated, DBA-lectin+ cells in the decidua basalis (DB) (Paffaro et al., 2002). By gd 10, uNK cells are numerous, reach peak numbers and are localized primarily to DB and the mesometrial lymphoid aggregate of pregnancy (MLAp). An acute onset of nuclear fragmentation occurs at gd 12 and uNK cells begin to decline in number and are rare by term (Peel, 1989; Delgado et al., 1996; Paffaro et al., 2002).

Uterine NK cells are the only lymphocyte subset known to promote normal structural development within implantation sites where they participate in remodeling of maternal tissue (Croy and Kiso, 1993; Guimond et al., 1997). Uterine NK cells are essential for maintenance of integrity of DB during the second trimester of pregnancy, although they are not involved in initiating decidualization in mice (Greenwood et al., 2000). Murine uNK cells also trigger events promoting pregnancy-induced, spiral artery modification. Uterine NK cells produce the cytokine interferon (IFN)-γ, which regulates genes mediating thinning of these arterial walls, as well as dilation and elongation of these specific arterial segments (Ashkar & Croy, 1999; Ashkar, Di Santo and Croy, 2000; Croy et al., 2002). Human uNK cells are likely to have an analogous

function as they express genes for endothelial cell mitogens and other angiogenic factors (Langer, Beach and Lindenbaum, 1999; Li et al., 2001).

Differentiation of uNK cells depends upon E primed and P4-promoted changes in the endometrium termed decidualization (Finn, 1966; Peel, 1989). Whether E has direct receptor-mediated actions on uNK cells or their precursors or acts indirectly through other ER+ cell types known to be present in the uterus (Souse & Korach, 1999), remains controversial. Using RT-PCR, we previously reported that cytolytic hybridoma cells formed between precursors of uNK cells and SP2/0 cells expressed ER (van den Heuvel et al., 1996). Two ERs, α and β, are now known (reviewed in Nilsson S et al., 2001) and the primers used in analysis of the hybridoma cells were for ERα. Recent studies using immunohistochemistry suggest that mouse NK splenic cells functionally express ERα and ERβ (Curran et al., 2001). This supports earlier work of Gruber and colleagues (1988) who reported that mouse splenic NK cell lytic activity varied with stage of the estrous cycle. Highest lytic units were found in E-dominated pro-estrous and estrous stages. It is also consistent with reported estradiol (E2)-regulated killing of mouse tumor targets in vitro and in vivo by steroid-treated, activated NK cells (Baral, Nagy and Berczi, 1995). ERα is expressed mainly in female organs, such as the ovary, uterus, vagina and mammary gland but ERα is also found in other tissues including nervous, osseous and cardiovascular (Muramatsu and Inoue, 2000; Nilsson et al., 2001). ERβ is strongly expressed in ovary, lung and prostate. ERβ is also expressed in testicular germ and Sertoli cells of rodents, ducts of the male urogential system, hypothalamus and cerebral cortex and in the cardiovascular system (Mosselman, Polman and Dijkema, 1996; Hess et al., 1997; Tremblay et al., 1997; Nilsson et al., 2001). The two ERs display 96% homology in their DNA binding sequence and activate transcription of similar

genes. The ERα and ERβ ligand binding domains share only 53% homology, but exhibit similar binding affinities for endogenous estrogens (i.e. estradiol, estrone). ERβ has a binding preference for natural plant products and metabolites such as genistein, compared to ERα. Both ERs are members of the nuclear receptor family that includes receptors for thyroid hormone and vitamin D (Muramatsu and Inoue, 2000).

ER expression has been studied on NK cells of rats and humans. Ogle and his colleagues (1997) found expression of multiple isoforms of ER and PR by immuno-histochemistry in DB of rat implantation sites. Careful attention was paid to uNK cells but lymphoid cells were found to be devoid of both R. In contrast, Baral et al. (1997) found that E-antagonists (tamoxifen and toremifene) promoted lytic activity of activated rat NK cells against ER-negative tumor cells in vivo. Using the same antibodies to ER and PR in independent immunohistochemical studies of normal and pathologic early human gestational materials, two groups have reported abundant expression of ER and PR by stromal and glandular cells but not by uNK cells (Jones, Bulmer and Searle, 1995; 1998; King, Gardner and Loke, 1996). Others also described uterine cells expressing ER as stromal but have not indicated specifically that lymphocytes were unreactive (Snijders et al., 1992 (human); Tibbets et al., 1998 (mouse)). However, in vitro studies of a human NK cell line showed that E2, but neither P4 nor testosterone, stimulated proliferation and lytic activity and that these effects were blocked by tamoxifen (Sorachi et al., 1993).

Zhang et al. (1999), evaluating NK cells isolated from human blood in vitro, found that isoflavones, available from dietary soybeans, had weakly estrogenic effects and activated lytic activity.

Mice ablated for ERα (αERKO) and ERβ (βERKO) (Korach, 2000) have been invaluable in distinguishing different roles for each R in E-mediated signaling (Couse and Korach, 1998, 1999). The reproductive phenotypes of αERKO and βERKO mice are different. The αERKO is infertile in both sexes, displays abnormal sexual behavior and has impaired reproductive organ development. In contrast, βERKO female mice are subfertile and have decreased ovarian function but exhibit normal sexual behavior and conceive. Litter sizes are smaller than for the congenic strain. (Muramatsu and Inoue, 2000). Male βERKO mice are fertile and exhibit normal sexual and reproductive behaviour. To assess the whether precursors of uNK cells express ERα or ERβ during recruitment to the uterus and terminal differentiation, bone marrow (BM) was extracted from αERKO and βERKO mice and transplanted into immunodeficient RAG-$2^{o/o}$ × common cytokine receptor chain γ (γc) $^{o/o}$ (RAG-$2^{o/o}$/$\gamma c^{o/o}$) mice (Colucci et al., 1999). The engrafted mice were subsequently mated. RAG-$2^{o/o}$/$\gamma c^{o/o}$ mice lack all lymphocyte lineages and have well characterized implantation site pathology that is reversed, in a quantifiable manner, by adoptive transfer of murine marrow containing uNK precursor cells (Guimond, Wang and Croy, 1998; Ashkar, Di Santo and Croy, 2000). The objective of this experiment was to assess the homing and differentiation potential of uNK precursor cells that genetically lack ERα or ERβ.

MATERIALS AND METHODS

Mice and Bone Marrow Transplantation

Adult female αERKO (n=5) and βERKO (n=5) mice were shipped from NIEHS to University of Guelph for experimental study. C57Bl/6J (B6) females were purchased from Jackson Laboratory, Bar Harbor, ME. RAG-$2^{o/o}/\gamma c^{o/o}$ mice (Colucci et al., 1999) were bred and housed under pathogen-free barrier husbandry at the University of Guelph. All animal procedures were conducted under approved Animal Utilization Protocols. Recipient female RAG-$2^{o/o}/\gamma c^{o/o}$ mice (n=10) were preconditioned using two intraperitoneal injections of 5-fluorouracil (5-FU, 150 mg/kg), at 48 and 24 hr prior to BM infusion. Cells ($2x10^6$), obtained from the pooled long bones of either αERKO or βERKO females, were infused into the preconditioned RAG-$2^{o/o}/\gamma c^{o/o}$ recipients by the lateral tail vein. After 3 wk, recipients were paired with RAG-$2^{o/o}/\gamma c^{o/o}$ males for mating and seven females became pregnant. Negative control mice were gd-matched RAG-$2^{o/o}/\gamma c^{o/o}$ receiving PBS. Positive control mice were gd-matched B6 whose implantation sites have been previously documented to be identical to those in RAG-$2^{o/o}/\gamma c^{o/o}$ mice receiving $2x10^6$ B6 BM cells (Ashkar et al., 2000). Gestation day was calculated from recognition of a copulation plug, called gd 0. Recipients and control mice were euthanized using CO_2 at gd 10 or 12.

Processing and Analysis of Paraffin Embedded Tissues

Immediately following asphyxiation, pregnant mice were slowly perfused over a period of 15 minutes with 12 ml 4% paraformaldehyde in 0.1 M PBS/0.1 M sucrose into the dorsal aorta. Uteri were then removed and most implantation sites were processed into paraffin using standard methodology. Litter sizes were 5, 6 and 7 for recipients αERKO BM and two each of 2 or 5

fetuses for recipients of βERKO BM. Two or three implantation sites from each pregnant recipient (two females per study group except αERKO BM transplanted gd 10, for which a single female with 6 implant sites was available) were serially sectioned at 7μm and stained using hematoxylin and eosin (H&E) or Periodic acid-Schiff (PAS) reaction. The number of uNK cells/mm^2 of tissue was measured as previously described (Ashkar and Croy, 1999; Chantakru et al., 2001). Eleven PAS-stained sections from the center of each implantation site were enumerated. The sections were separated by 49 μm to avoid duplicate counting of large, mature uNK cells. DB and MLAp were scored as independent microdomains separated by the circular smooth muscle of the uterine wall. Counts were made using an ocular grid at 500x magnification. For each microdomain, the 33 or 44 estimates of uNK cell frequency were averaged. Ratios for blood vessel area/lumen area of decidual spiral arteries, cut in cross section were also measured on 11 H&E stained sections (adjacent to the PAS stained sections) from each implant site studied. Morphometry measurements were made using Optimas 6.2 (Optimas Corporation, Bothwell, MA).

Ultrastructural Processing and Analysis

For ultrastructural studies, mesometrial decidua generated in 6 implant sites from αERKO BM recipients and 4 implant sites from βERKO BM recipients, was cut into small pieces and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer for 3-4 hr at 4° C, washed in 0.1M phosphate buffer and postfixed in 1.5% osmium tetroxide, washed again, dehydrated in ethanol and propylene oxide and embedded in Jembed Resin 812 (Canemco Inc., Montreal, PQ). Semithin sections of 1.0 μm thick were cut (Sorvall MT2-B ultramicrotome) and

stained with 1% toluidine blue. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, examined and photographed with JEOL-100S transmission electron microscope.

Magnetic Bead Isolation of uNK cells, RNA Isolation and RT-PCR for ER and β actin

UNK cells were isolated on 4 occasions from PBS-perfused B6 mice at gd 10 (n=2), 12 and 13 using Dolichos biflorus (DBA) lectin (Sigma Co., St. Louis, MO) conjugated to 4.5μm magnetic beads (CelLection Biotin Binder Kit, Dynal, Lake Success, NY), using a protocol developed by Lima (2002) from the manufacturer's recommendations. Briefly, DB and MLAp from each implantation site were separately dissected, pooled by litter and very finely minced in 1 ml of RPMI medium containing 1000 IU DNase and 2% bovine serum albumen (Gibco, Grand Island, NY). Tissue fragments were pipetted up and down using a 1 ml pipette, mounted with a disposable tip, 10-15 times to further dissociate the fragments. The suspension was passed over nylon 80-mesh screen to recover dissociated cells that were then exposed to DBA lectin-conjugated magnetic beads (4 ^{0}C, 30 min with gentle shaking). After exposure of the suspension to magnetic forces, uNK cells were washed (x3) with PBS, detached into PBS by adding 0.1M N-acetyl D-galactosamine (Sigma, St. Louis, MO), the specific sugar ligand of DBA lectin, and used immediately for RNA isolation. To establish purity of the prepared cells, aliquots were examined by differential interference contrast microscopy for general features and by FITC-labelled DBA lectin staining and fluorescence microscopy for uNK cell identification. At gd 10 and 12, implantation sites from other B6 females were also dissected, using dissection microscopic magnification, to recover DB and MLAp as intact tissue for RNA analysis. Ovaries and uteri from two virgin B6 mice were used to isolate control RNA. Total RNA was isolated

using the *RNeasy Mini Kit* (Qiagen, Mississauga, ON) according to manufacturer's instructions. A *First-strand cDNA Kit* (Amersham Pharmacia Biotech, Baie d'Urfé, QU) was used to generate cDNA, in a 33 µl mixture containing 2.5 g total RNA, 11 µl bulk first-strand cDNA mix, 1µl DTT, 1ul primer Not-I-d $(T)_{18}$ diluted 1:25 in DEPC H_2O. The reaction was incubated (37°C, 1h) followed by 5 min. at 90°C to inactivate the enzyme. One µl of cDNA was then subjected to PCR in a 15µl volume containing 11.555µl DEPC H_2O, 1.5µl PCR Reaction Buffer 10 x (Sigma), 0.3µl dNTP (Amersham Pharmacia Biotech), 0.045µl Taq DNA Polymerase (Sigma) and 0.3µl of primers sense (ERα-ACCATTGACAAGAACCGGAG; ERβ-GAAGCTGGCTGACAAGGAAC; antisense (ERα-AAGGACAAGGCAGGGCTATT; ERβ-AACGAGGTCTGGAGCAAAGA. The primers GCTACAGCTTCACCACCACA and ACATCTGCTGGAGGTGGAC were used as controls for amplification of mouse β-actin. The predicted sizes of the amplicons are 248 bp for ERα, 187 bp for ERβ and 477 bp for mouse β-actin. The cycling parameters were: 95°C, 56°C and 72°C for 60 seconds each for a total of 35 cycles. The PCR products were separated and sized on 1% agarose ethidium bromide gels. To confirm amplification of the correct target sequence, amplicons for ERα and ERβ were cloned and sequenced (University of Guelph, Guelph Molecular Supercentre).

Statistics

The means and standard deviations of uNK cell/mm^2 were calculated and compared to paired, gd-matched controls for the same microdomain (DB or MLAp). Vessel area to lumen area ratios were also compared to those for the matched control. Statistical comparisons were made using ANOVA (SAS 6.12; SAS Institute, Cary, NC) Significance was considered at $p<0.5$.

RESULTS

General Features of Implantation Sites in RAG-2$^{o/o}$/γc$^{o/o}$ Recipients of ERKO BM

Implantation sites of RAG-2$^{o/o}$/γc$^{o/o}$ females engrafted with αERKO or βERKO BM appeared normally developed and similar to those in B6 mice at gd 10 (Fig. 1A and B compared to D) and at gd 12 (not shown). The DB was more cellular than in gd-matched RAG-2$^{o/o}$/γc$^{o/o}$ receiving PBS (Fig. 1C) and the MLAp formed in the uterine wall. PAS+ uNK cells were identified in both DB and MLAp of all BM recipients but not in the uteri of PBS-treated RAG-2$^{o/o}$/γc$^{o/o}$ females.

Assessment of uNK Cell Numbers and Morphology in RAG-2$^{o/o}$/γc$^{o/o}$ recipients of ERKO BM

UNK cells are absent from the implantation sites of non engrafted RAG-2$^{o/o}$/γc$^{o/o}$. Numbers of uNK cells at midgestation in DB and MLAp of grafted mice and B6 congenic controls are presented in Fig. 2. UNK cell counts in implantation sites from recipients of either αERKO BM or βERKO BM were not significantly different from counts in control B6 sites at either gd studied. More uNK cells were found in MLAp compared to DB, and gd 10 counts were higher than gd 12 counts. Each of these observations was consistent with findings in the B6 control mice and with previously reported results (Ashkar and Croy, 1999; Chantakru et al., 2001). This indicates that uNK precursor cells, from ERKO mice, home to the uterus and localize to appropriate microdomains within implantation sites as well as precursors from wildtype mice.

UNK cells derived from α and βERKO BM showed all stages of differentiation from poorly granulated to heavily granulated and the graft-derived uNK cells had perivascular and intravascular associations similar to uNK cells in B6 mice (Fig. 3). Ultrastructurally, graft-

derived uNK cells had asymmetric nuclei and their cytoplasm contained granules of various sizes with homogeneous core density and a cap of densely packed microvesicles. Cytoplasmic organelles, including mitochondria, rough endoplasmic reticulum and Golgi were prominent (Fig. 4), indicating typical uNK cells with high metabolic activity (Peel, 1989). Some uNK cells derived from either α or βERKO BM had a less organized cytoplasm that showed patchy granularity. This observation was consistent amongst recipients but was not considered of major significance given the abundance of cells with normal ultrastructural appearance.

Assessment of uNK Cell Activity in Implantation Sites of RAG-2$^{o/o}$/γc$^{o/o}$ Recipients of ERKO BM

The spiral arteries of RAG-2$^{o/o}$/γc$^{o/o}$ are not modified at midgestation. Transplantation of uNK cell progenitors genetically capable of IFN- γ production, or treatment of pregnant RAG-2$^{o/o}$/γc$^{o/o}$ females with mrIFN-γ induces modifications reflected histologically as decreased spiral artery wall area/lumen area ratios. To address whether graft-derived uNK cells, lacking ERα or ERβ, could trigger modification of the spiral arteries, morphometry of these decidual vessels was undertaken in graft recipients. Ratios of total vessel cross sectional area to total lumen cross sectional area were calculated and compared to ratios of the measurements made in gd-matched C57Bl/6J and PBS-treated RAG-2$^{o/o}$/γc$^{o/o}$ females. As shown in Fig. 5, the wall area:lumen area ratios of spiral arteries of the grafted mice were different from those in gd-matched, PBS-treated RAG-2$^{o/o}$/γc$^{o/o}$ females ($p<0.05$) and similar to those in B6 mice ($p>0.05$). These data confirm that pregnancy-induced arterial modification occurred after BM transplantation and that ER expression by uNK cells is not essential for uNK cell triggering of this physiological change or for its completion

RT-PCR for ER Expression by DBA-Lectin Purified uNK Cells from Normal Mice

A recently developed technique for rapid isolation of viable, highly enriched uNK cells (95%-100% purity, Fig. 6C-E) from mouse implantation sites was used to prepare uNK cells from B6 implantation sites on gd 10, 12 and 13. Totals of $2.5x\ 10^4$, $5x10^4$ and 2.5×10^4 cells were recovered per pregnant uterus respectively and immediately used for isolation of total RNA. Using RT-PCR and primers for β-actin, each uNK cell enriched preparation provided RNA of good quality (Fig 6B). Message for ERα was found in the DB and MLAp at both gd 10 and 12. However, RNA from uNK cells isolated at gd 10, 12 and 13 failed to demonstrate message for ERα, suggesting that cells other than uNK cells transcribe ERα in the DB and MLAp. Uterus and ovary from the virgin B6 mouse showed message for ERα. No samples of pregnant and virgin uteri showed expression of ERβ. ERβ expression was detected in ovary, the positive control tissue for ERβ (Fig. 6A).

DISCUSSION

Mature lymphocytes are not present in RAG-$2^{o/o}/\gamma c^{o/o}$ mice although the strain is fertile and vigorous under barrier husbandry conditions. Implantation sites in these animals have inappropriate development of maternal tissues forming the placental bed and contributing to the placenta. The inappropriate features are spiral arteries with high wall area to lumen area ratios, absence of uNK cells, absence of MLAp development and decidual hypocellularity with edema. These features can be quantified and can be reversed by transplantation of uNK precursor cells

capable of IFN-γ production. Following transplantation of BM from B6 mice, no features of the implantation sites in the subsequent pregnancy different from implantation sites in naturally mated, genetically normal B6 females (Ashkar et al., 2000). The arterial and decidual anomalies may also be normalized in the absence of uNK cells through inoculation of uNK cell produced or regulated products such as IFN-γ or α2-macroglobulin (Ashkar et al., 2000; Croy et al., 2002; Esadeg, 2002).

In the present study, BM from mice with a B6 background but lacking the gene for either ERα or ERβ was transplanted to RAG-$2^{o/o}/\gamma c^{o/o}$ mice and implantation sites in subsequent pregnancies were normalized.Uterine NK cells lacking either ERα or ERβ homed to the uterus appropriately and differentiated fully, as assessed by their location, number, granularity and morphology, including ultrastructure. Graft-derived uNK cells also appeared to function since actions on spiral arteries were quantified. These observations suggest that there is no direct regulation of uNK cells through ERα or ERβ and that the requirement for E priming during induction of uNK cell differentiation is mediated indirectly through receptor expression on other types of cells. Alternately, ERα and ERβ might act in a reciprocal manner to one another, such that when one receptor subunit is absent, the second receptor type would compensate for the loss of the first. However, this premise is somewhat unlikely because of the limited homology of ligand-binding sites (53%) which suggests binding of different ligands and because no ER mRNA was found in purified uNK cells. Mouse uNK cells maybe distinct from human uNK cells in this regard as re-examination of human uNK cells using Realtime-PCR has shown message for ERβ but not for ERα or PR (Critchley et al., 2002 and Critchley, Saunders, King, Henderson; personal communication).

Complex regulatory control is associated with ER-mediated effects (Nilsson et al., 2001), some of which might explain the essential requirement for E or E2 priming in mice for success in P4-mediated decidual induction and appearance of uNK cells. Uterine NK cells might be regulated through interactions with the aryl hydrocarbon receptor which has an unknown physiological ligand, use of estrogen receptor related (ERR) receptors α, β and/or γ (Sladek and Giguere, 2000; Coward et al., 2001), competition for shared cofactor binding, use of as yet undefined ERs or signalling via the epidermal growth factor (EGF) pathway. However, it has been shown that mouse uNK cells produce EGF while stromal cells in the MLAp express EGFR (Kusakabe et al., 1999), suggesting that E or E2-related signalling involving EGFR would occur in the stroma rather than in the lymphocytes.

Re-introduction of DBA lectin, as a reagent to identify murine uNK cells (Damjanov and Damjanov, 1992; Stewart and Webster, 1997), has been important for easy histological definition of early, non-granulated uNK cells in implantation sites (Paffaro et al, 2002). DBA lectin histochemistry, in contrast to PAS, reveals the uNK cell membrane. Both reagents identify uNK cell cytoplasmic granules, which are prominent from gd 7. DBA-lectin histochemistry of wildtype mice at gd 10 reveals extended cytoplasmic processes of proliferating uNK cells in the mesometrial myometrium where abundant stromal ER+ cells have been found by in situ hybridization (Tibbitts et al., 1998). Proximity of the two cell types suggests that the E/E2 mediated gene regulation triggering terminal uNK cell differentiation could occur in stromal cells. Changes in stromal cell gene expression, surface properties and/or secreted products could activate neighbouring uNK cell precursors.

DBA-lectin has also offered the first truly successful rapid approach for mouse uNK cell isolation that yields high quality RNA. Murine uNK cells have not been cloned or derived as cell lines; primary cell isolates undergo rapid apoptosis, making preparation of RNA from isolated cells of questionable value (Delgado et al., 1996; Croy et al., 1997). RNA prepared from uNK cells rapidly isolated using DBA-lectin conjugated magnetic beads was not degraded and was suitable for preparation and analysis of cDNA. The DBA-lectin isolated cell suspensions appear to be ≥95% uNK cells, the range of cell purity achieved by flow sorting of human CD56+ cells from early human decidua (Jokhi et al., 1994; Jones, Bulmer and Searle, 1997). Perfusion of the mice prior to dissection of the mesometrial tissue elevates purity to approach 100%. Availability of RNA from non-cultured mouse uNK cells should now permit rapid advances in understanding the regulation of this cell lineage and its functions in the mesometrial decidua.

ACKNOWLEDGEMENTS

We thank Ms. Kanmei Lui for her assistance in preparation of the histology, Dr. J.P. Di Santo, Pasteur Institute, Paris, for providing breeding stocks of RAG-$2^{o/o}/\gamma c^{o/o}$ mice and Drs. W. Foster, Hamilton, ON, M. van den Heuvel and J. Raeside, Guelph, ON for helpful discussions. These studies were supported by Awards from the Ontario Graduate Scholarship Program; Ontario Ministry of Agriculture, Food and Rural Affairs; the Natural Sciences and Engineering Council, Canada; an OVC Bull Research Travel Fellowship; a Royal Thai Government Scholarship and Fundacao de Amparo a Pesquisa do Estado de Sao Paulo.

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FIGURE LEGENDS

Fig.1. Photomicrographs of midsagittal sections of gd 10 implantation sites from RAG-$2^{o/o}/\gamma c^{o/o}$ recipients of αERKO BM, (A) RAG-$2^{o/o}/\gamma c^{o/o}$ recipients of βERKO BM (B) RAG-$2^{o/o}/\gamma c^{o/o}$ recipients of PBS (C) and C57Bl/6J congenic mice (D). In all images, the top represents the region of the mesometrial lymphoid aggregate in the uterine wall (MLAp) and the bottom, the placenta (P). In A, B and D, regions containing numerous uNK cells (arrows) are seen in the decidua basalis (DB) and MLAp. Bars represent 200 μm. Insets show boxed regions of the DB containing darkly stained uNK cells at higher magnification. Bars in the insets are 100 μm. UNK cells are missing in (C). PAS staining.

Fig. 2. Histogram depicting the mean numbers of uNK cells/mm^2 found in the MLAp and DB of RAG-$2^{o/o}/\gamma c^{o/o}$ recipients of αERKO BM, βERKO BM and of C57Bl/6J congenic mice at gd 10 and 12. There are no uNK cells in pregnant RAG-$2^{o/o}/\gamma c^{o/o}$ females who have not received lymphocyte precursor transplants.

Fig. 3. Photomicrographs of 1.0 μm semi-thin sections of decidual arteries at gd 10 and 12. (A and B) depict C57Bl/6J (B6) at gd 10 and 12 respectively and show gestationally modified vessels. (C) depicts gd 10 in an non-engrafted RAG-$2^{o/o}/\gamma c^{o/o}$ and illustrates an unmodified artery. The thick arterial wall is labeled AW and the arterial lumen, which is relatively constricted, is mark as *. Uterine NK cells are present in B6 and many (arrows) are closely associated with vessels (A and B). No uNK cells are present in RAG-$2^{o/o}/\gamma c^{o/o}$ implantation sites (C). (D and F) show spiral arteries in RAG-$2^{o/o}/\gamma c^{o/o}$

recipients of αERKO bone marrow at gd 10 and 12 respectively while (E and G) show spiral arteries in RAG-$2^{o/o}/\gamma c^{o/o}$ recipients of βERKO bone marrow at gd 10 and 12 respectively. UNK cells are intramural (arrowheads) as well as perivascular (arrows) and intravascular (*). In comparison to the artery in (C), pregnancy induced vascular remodeling has occurred in the RAG-$2^{o/o}/\gamma c^{o/o}$ receiving marrow grafts. The uNK cells in F and G, (gd 12) are larger and show heavier cytoplasmic granulation than in (D and E; gd 10), which is typical of later uNK cell differentiation. In comparison with (C), the arterioles in the recipients of βERKO BM are modified. Toluidine blue stained, bars = 50 μm.

Fig. 4. Electron micrographs of uNK cells from RAG-$2^{o/o}/\gamma c^{o/o}$ recipients of αERKO BM (A,C), a RAG-$2^{o/o}/\gamma c^{o/o}$ recipient of βERKO BM (B) and C57Bl/6J congenic mice (D). Tranplant-derived uNK cells are typical in appearance compared to the normal control (D) and characterized as having membrane-limited cytoplasmic granules of various sizes that have homogeneous cores and caps packed with microvesicles. Transplant-derived uNK cells show an abundance of rough endoplasmic reticulum (rER) and mitochondria (m). In (C), the close association of a graft-derived ERα-deficient uNK cell of normal appearance (arrow) is shown in a normal intramural position adjacent to an endothelial cell lining a maternal vessel. BV-blood vessel, E-endothelium. Sections stained with uranyl acetate and lead citrate. Bars are 1.6 μm in A&D, 2μm in B and 2.6 μm in C.

Fig. 5. Histogram presenting summary of vessel area to lumen area ratios for the spiral arteries of RAG-$2^{o/o}/\gamma c^{o/o}$ recipients of αERKO BM, βERKO BM or PBS and of C57Bl/6J congenic mice at days 10 and 12 of gestation. The ratios for the engrafted mice did not differ at either gd from the genetically normal mice but were different to the ratios for the non-engrafted RAG-$2^{o/o}/\gamma c^{o/o}$ at each gestation day.

Fig. 6. Ethidium bromide stained gels showing amplicons for (A) ERα (upper part) and ERβ (lower part) and (B) for β-actin derived from RT-PCR reactions using tissues and cells from C57Bl/6 mice. Lane 1 is a 100 bp DNA ladder. Lanes 2, 6 and 10 are blank while Lane 13 shows the water amplification negative control for each primer set. Lanes 3, 4, and 5 show products from cDNA of gd 10 DB, MLAp and DBA-lectin purified uNK cells respectively. Lanes 7, 8, and 9 show products from cDNA of gd 12 DB, MLAp and DBA-lectin purified uNK cells respectively. Lane 11 represents cDNA from virgin uterus and Lane 12 cDNA from ovary of a virgin adult mouse. Message for ERα (248 bp) was detected in DB and MLAp at gd 10 and 12 but not in isolated uNK cells at these days nor at gd 13 (not shown). Sequencing was used to confirm the identification of the 100%-matched amplicon. Message for ERβ (187 bp) was detected only in ovary (Lane 12), a tissue known to express ERβ (Nilsson et al., 2001). Identity of this amplicon was also confirmed by sequencing. All cDNA samples were transcribed successfully for mouse β-actin in the same experiment and in additional experiments.

Panels C-E show the steps in preparation of the highly enriched uNK cell population from dissociated DB of a perfused mouse using DBA-lectin

conjugated magnetic beads. (C) illustrates the dissociated decidual cells (arrows) mixed with the lectin-labelled beads (arrowheads) under differential interference contrast (DIC). Bar represents 15 μm. (D) shows DIC imaged cells isolated following application of the magnet and then release of PBS-washed cells from the beads by incubation with the competing sugar, N-acetyl galactosamine. Bar represents 10 μm. (E) shows immunofluorescent staining of the recovered lymphocytes using FITC-tagged DBA lectin, which reacts only with uNK cells (Paffaro et al., 2002). Bar represents 30 μm.

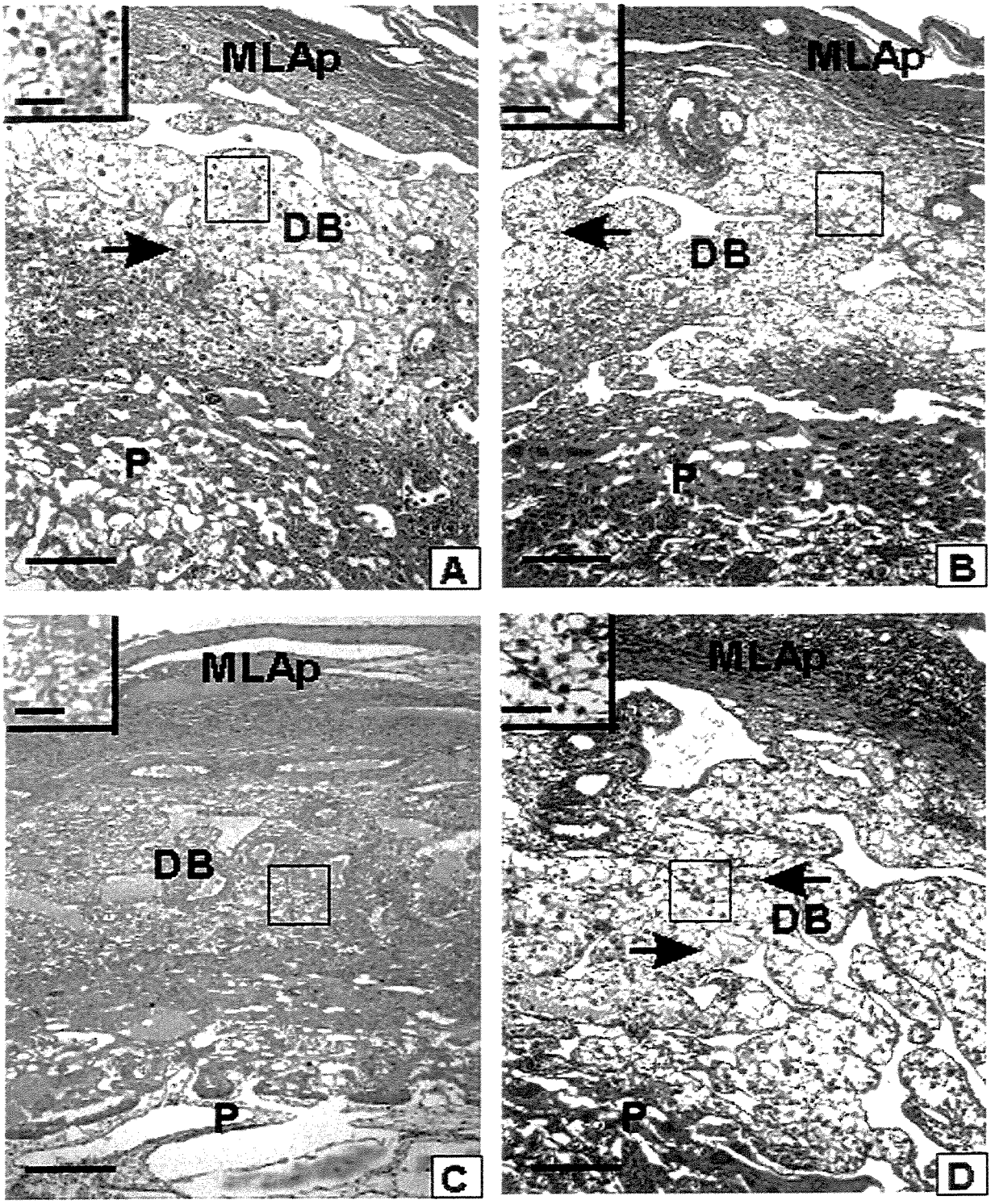


Figure 1

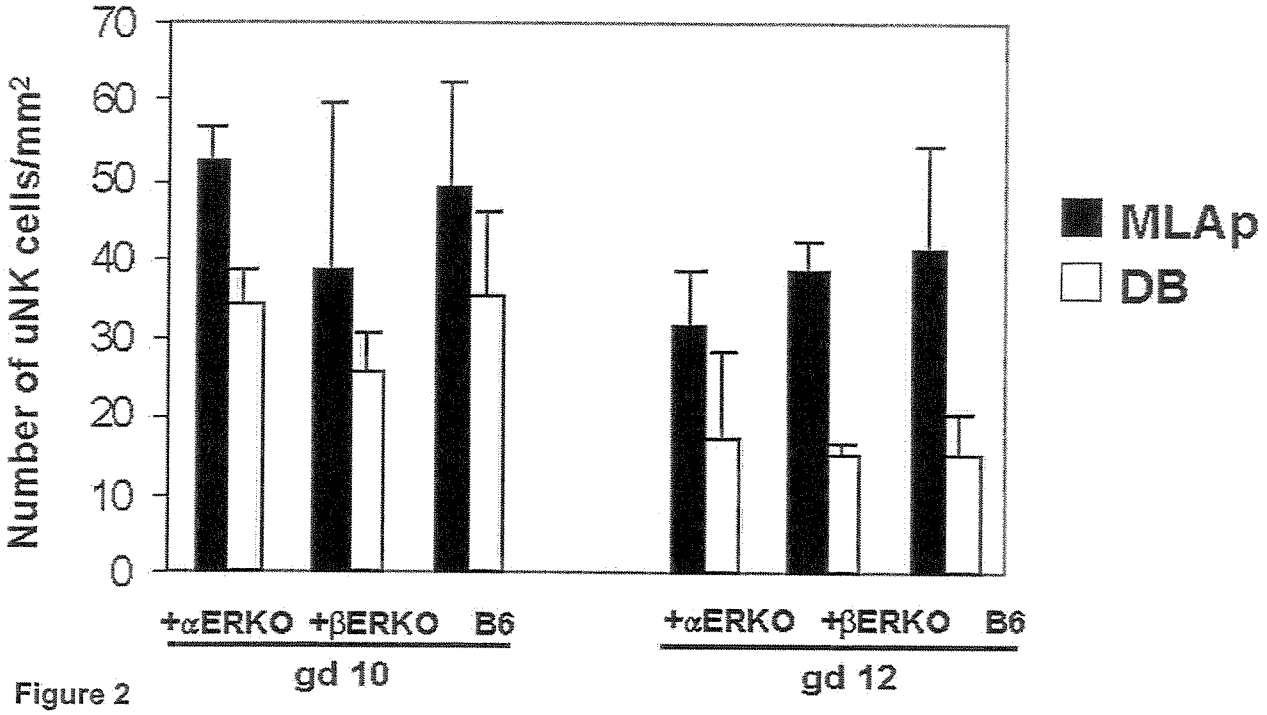

Number of uNK cells/mm2
70
60
50
40
30
20
10
0
+αERKO
+βERKO
B6
gd 10
+αERKO
+βERKO
B6
gd 12
MLAp
DB

Figure 2

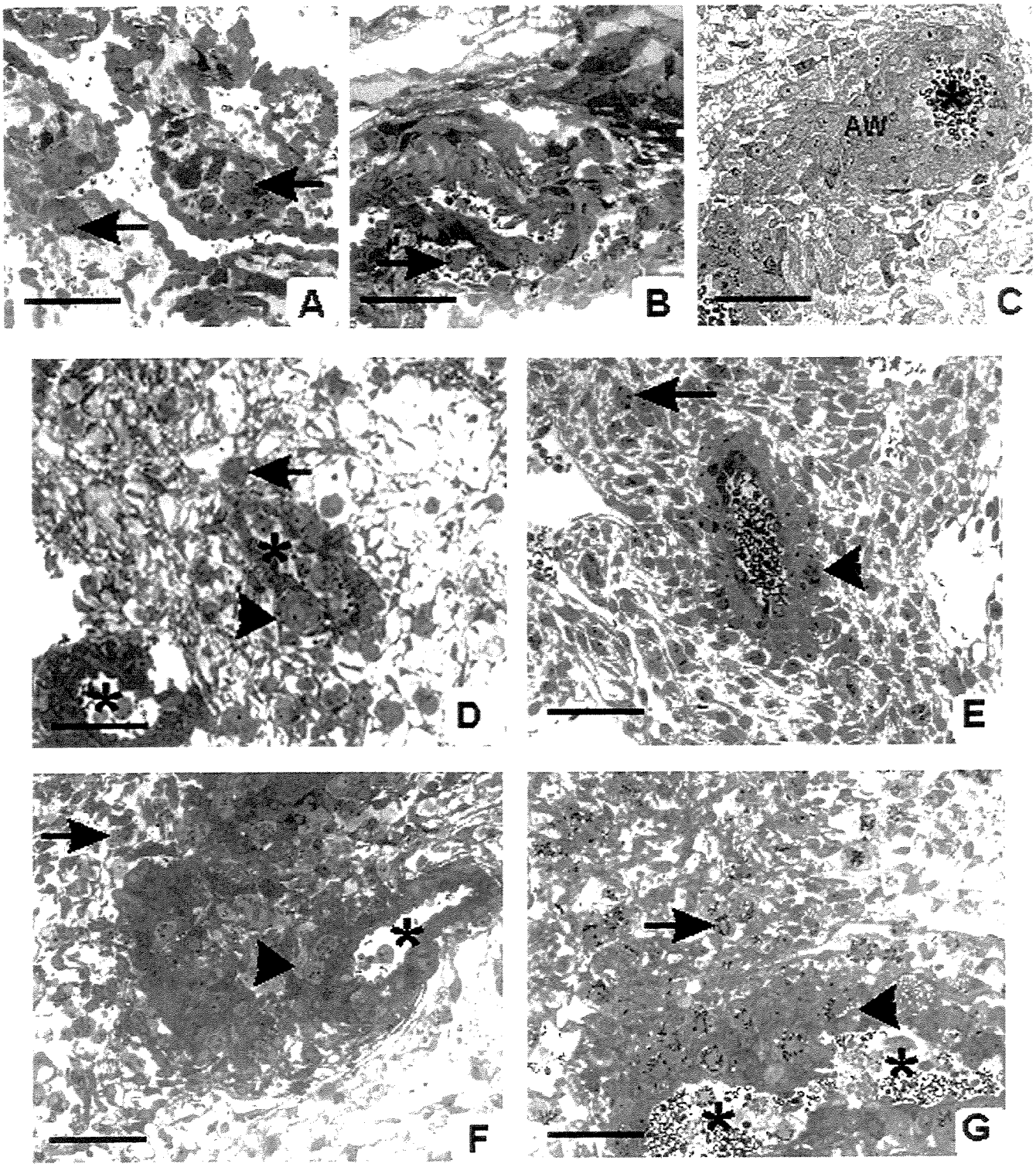


Figure 3

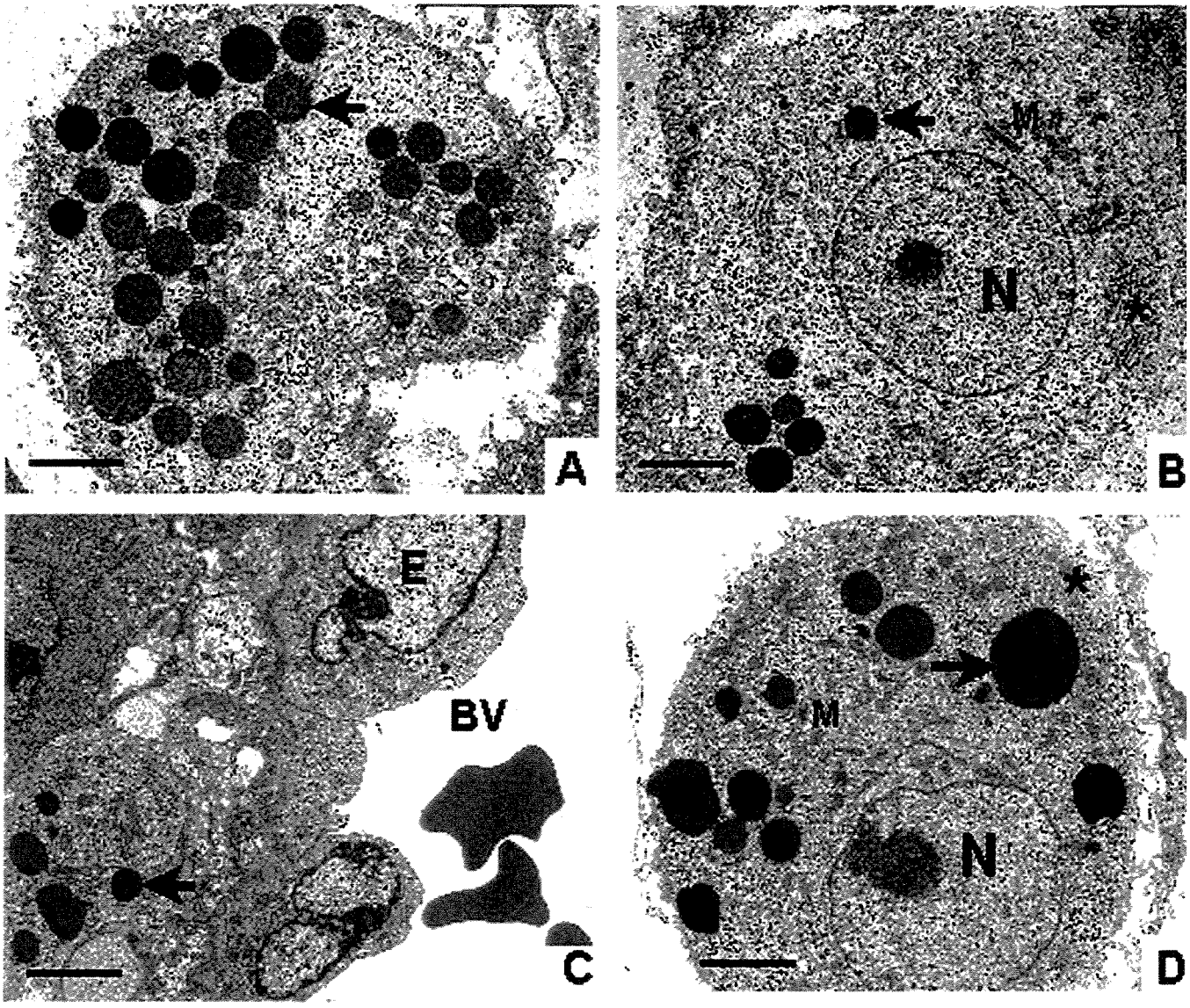


Figure 4

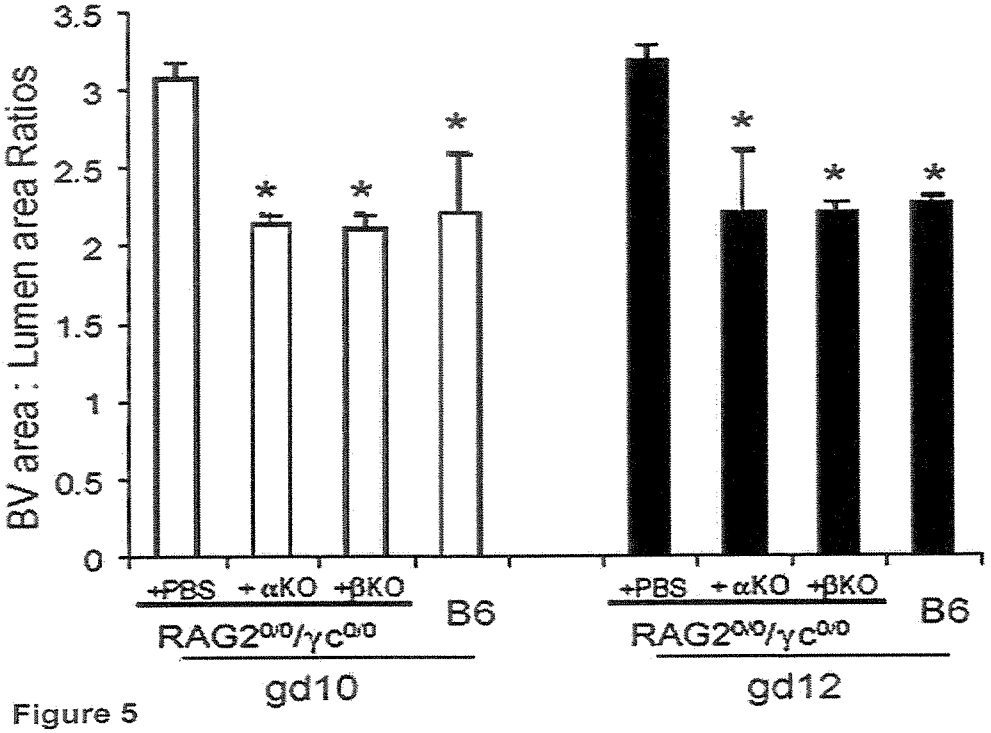
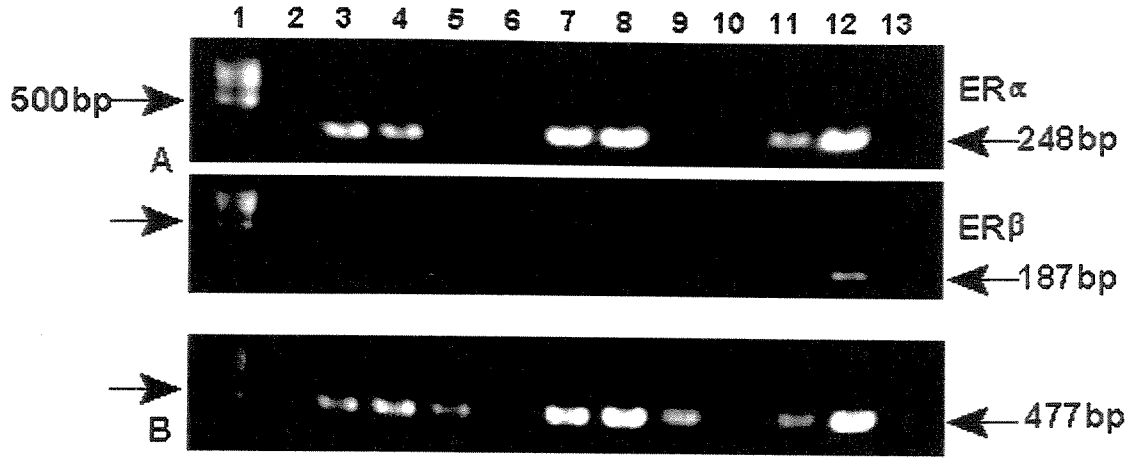

BV area : Lumen area Ratios
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
*
*
+PBS
+αKO
+βKO
B6
RAG2α/0/γcα/0
gd10
+PBS
+αKO
+βKO
B6
RAG2α/0/γcα/0
gd12

Figure 5



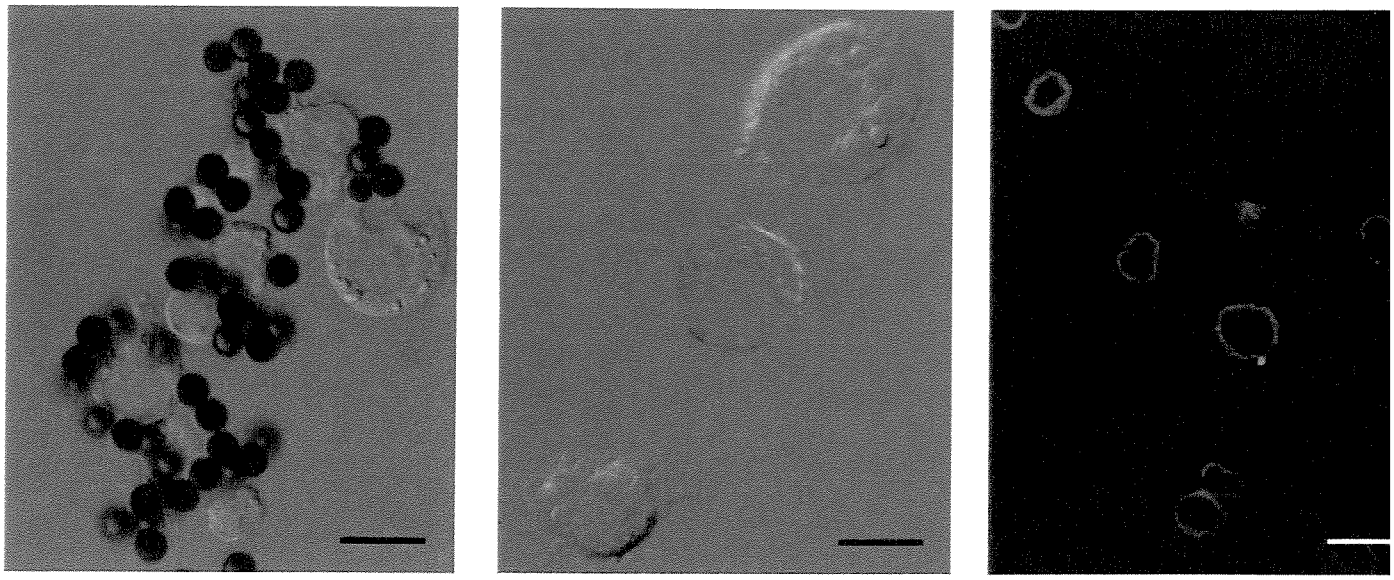


Figure 6

CAPÍTULO IV

LY49 Gene Expression by Natural Killer Cells Localized to Mouse Uterus[1]

Valdemar A. Paffaro Jr[*] [†], Hong He[*], Aureo T. Yamada[†] and B. Anne Croy[*,2]

[*]*Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada, N1G 2W1, and* [†]*Department of Histology and Embryology. Institute for Biology. UNICAMP Campinas, Sao Paulo, Brazil. 13083-970*

Running Title: LY49 expression by uterine lymphocytes.

Keywords: Rodent, NK cells, Reproductive Immunology, Cell Differentiation, Cell Activation.

1. Supported by Natural Sciences and Engineering Council, Canada, Ontario Ministry of Agriculture, Food and Rural Affairs and Fundacao de Amparo a Pesquisa do Estado de Sao Paulo.

2. Address correspondence to Dr. Anne Croy, Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

E-mail address: acroy@uoguelph.ca;

Phone: 519-824-4120, x4915;

FAX: 519-767-1450

3. Abbreviations used in this paper: DB, decidua basalis; DBA, *Dolichos biflorus* agglutinin; gd, gestation day; MLAp, mesometrial lymphoid aggregate of pregnancy; MT, mesometrial triangle; PFA, paraformaldehyde; u, uterine.

ABSTRACT

Abundant numbers of NK cells infiltrate decidualizing mouse uteri, localize to specific microdomains and associate with arteries supplying placentae. Between gestation days (gd^3) 6-12, uterine (u)NK cells undergo four morphologically characterized steps of terminal differentiation. To investigate whether uNK cells are a selected NK cell subset and whether they display receptors in patterns coinciding with morphologic differentiation, expression of 14 LY49 receptors and DAP12 was analyzed by RT-PCR in dissected mesometrial tissues. C57Bl/6J (B6), randombred (predominantly $H\text{-}2^b$) *scid/scid*, B6-IL-$15^{-/-}$ and B6 *nu/nu* virgin uteri expressed DAP12 plus a partial repertoire. Syngeneic pregnancy induced additional expression of inhibitory receptors in all strains. In virgin B6 uteri, LY49D and LY49H activation receptors were co-expressed with inhibitory LY49I, J and Q. Early pregnancy (B6, gd6) induced full repertoire expression prior to morphologic maturity of uNK cells. At midgestation (B6, gd10), maternal decidua basalis, mesometrial lymphoid aggregate of pregnancy and fetal placenta expressed full repertoires. Perfusion (B6, gd10) revealed partial loss of repertoire from placenta, identifying lymphocytes expressing LY49A, C, F and/or I as a subset potentially interactive with intravascular fetal trophoblasts, cells active in physiological modification of segments of the maternal arterial tree during pregnancy.

INTRODUCTION

A transient lymphocyte population appears in pregnant uteri (primates and rodents) during decidualization, a gestational process that transforms uterine stromal cells into the maternal placental compartment. Murine transplant models have shown that these pregnancy-associated, NK lineage cells differentiate from progenitors found in lymphohematopoietic tissues, particularly spleen and lymph nodes (1). In mice, uterine Natural Killer (uNK) cells are first detected using *Dolichos biflorus* (DBA) lectin histochemistry, at gd4, the day of implantation, as rare, small, non granulated cells in decidua basalis (DB; 2). By gd5, uNK cell differentiation commences with some cells acquiring DBA lectin+ cytoplasmic granules. Gd6 is the earliest time at which significant numbers of uNK cells are found histologically and they are localized to, rather than between, implantation sites, along the mesometrial side of the uterus. The mesometrium (broad ligament) is the conduit for uterine vessels and nerves. By gd6, cell division and IFN-γ production are also initiated (3). Gd7, is the earliest day that four distinct subsets of DBA lectin + uNK cells are present. Peaks in both uNK cell numbers and in production of IFN-γ occur at gd10. Thereafter, cell division ceases and the population declines (2,4,5). Women have analogous CD56+, CD16-, CD3- cells that appear in the decidualizing uterus during the late secretory phase of menstrual cycle and early pregnancy and then decline (6).

Early murine uNK cells are recognized by immunohistochemistry as LY- 49G2^{+} cells (7-9). This marker declines about gd8 and asialo-GM1^{+} is displayed at the cell surface as the cytoplasm accumulates glycoprotein rich granules (8,9). The granules contain perforin, serine esterases, phosphatases, other enzymes and mucin (10-12). Phenotypic assignment of pregnancy associated granulated lymphocytes to the NK lineage is supported by their presence in *scid/scid* (T^{-}, B^{-}, NK^{+}), *nu/nu* and IL-$2^{-/-}/\beta_2^{-/-}$ mice and absence from tgε26 (NK^{-} T^{-}), common cytokine chain gamma (γc)

deleted and alymphoid (Rag-$2^{-/-}/\gamma c^{-/-}$) mice (13-16).

Neither murine nor human uNK cells display robust lytic activity in culture (5,6). Mouse uNK cells trigger physiological modification in decidual spiral arteries and maintain decidual tissue integrity via IFN-γ release (3,17). UNK cells are highly localized to the central decidua basalis (DB), where many (>30%) are intravascular or associated with arterial walls (18). At each implantation site, uNK cells also contribute to development of a mural microdomain, rich in immature stages (2,10,15). These transient mural structures are known as the mesometrial lymphoid aggregates of pregnancy (MLAp; previous called the metrial gland). Human uNK cells may also function in vascular modification because they produce angiogenic molecules (19,20). In addition, human uNK cells are thought to have KIR-regulated interactions with trophoblast cells, the cells of the fetal placenta (21-23).

Murine NK cell receptors belong to the LY49 family of type II lectin glycoproteins and to the Ig family of CD94 molecules (24). The latter are also found in humans. All three families of NKR recognize MHC I (24-27). KIRs recognize the classical MHC I while NKG2/CD94 receptors bind human HLA-E or mouse $Qa1^b$, non-classical MHC-like molecules (28,29). B6 mice have 14 functional LY49 genes and five pseudogenes encoded on chromosome 6 (24,26,30-33). Multiple LY49 receptors are used by individual NK cells. A given LY49 gene is randomly selected and only expressed by a subset of NK cells. Once an NK cell receptor (LY49, KIR or CD94/NKG2) is expressed, it is stably maintained on the cell and on its progeny (34,35). There maybe distinct windows of opportunity when only certain receptors are available for selection. After adoptive cell transfer or culture, NK cells expressing LY49A or LY49G2 initiate expression of other LY49 receptors (34,36). This suggests either ordered development of LY49 receptors in the sequence LY49A only → LY49G2 only → LY49C/I, etc. or universal availability of receptors followed by progressive loss in genes available for selection (i.e. LY49A/G2/C/I → LY49 G2/C/I →LY49C/I). Another study, using early NK-committed progenitors

cultured in cytokines and RT-PCR analyses, suggests LY49B is expressed first, followed by LY49G, LY49C and then LY49A, D, E and F (37).

MHC class I binding to LY49A, C or G inhibits NK cell cytotoxicity while binding to LY49D activates cytotoxicity. LY49D and H associate with an activating molecule, DAP12 (38,39). Approximately 10% of murine $CD8^+$ $\alpha\beta$ TCR^+ T cells express at least one inhibitory LY49 receptor but LY49 expression on conventional $CD4^+$T cells is rare (40,41). Some NKT cells also express LY49 receptors (42-44), however the roles of LY49 receptors on T and NKT cells are less understood. The LY49 repertoire and its developmental time course are not defined for uterine lymphocytes. Mice provide accessible pregnancies with well-characterized stages of uNK cell differentiation and well-defined uNK cell localization within maternal and fetal parts of the implantation site and placenta. Analysis of LY49 expression was undertaken in B6 and B6-related immune deficient strains at gd6, the first day significant numbers of uNK cells are found in DB and at gd10, the day peaks in numbers of mature uNK cells and in functional activity (IFN-γ production and uNK cell-promoted spiral artery dilation) are reported.

MATERIALS AND METHODS

Mice. B6 mice (Jackson Laboratories, Bar Harbor, ME) were housed under conventional husbandry. Immunodeficient mice, (randombred ICR-*scid/scid* (about 90% H-2^b; NK+, T-, B-, Taconic, Germantown, NY), B6-*nu/nu* (NK+T-B+, Jackson Laboratories), B6-IL-$15^{-/-}$ (NK-; kindly provided by Dr Jacques Pleschon, Immunonex, Seattle, WA) were bred in the University of Guelph's barrier-husbandry facility. IL-$15^{-/-}$ mice lack NK cells and have marked reductions in thymic and peripheral T cells, memory CD8 cells, and subsets of intestinal intraepithelial lymphocytes (45). Four virgin B6-

RAG-2$^{-/-}$ females, provided by Dr. A. Ashkar, McMaster University, Hamilton, ON, were also used as unmated uterus donors. For all other strains, females at 8-12 wks of age were used as donors of virgin uteri or mated to males of their own genotype. The day a vaginal plug was detected was called gd0 and mated females were studied at specific timed days of gestation. Two gd10 B6 were also perfused with 60 ml PBS (20x total blood volume) prior to tissue collection. Euthanasia was by CO_2 followed by cervical dislocation. All procedures for animal handling were approval by the University of Guelph Animal Care Committee.

Tissue Dissection, RNA Isolation and PCR. Virgin uterus, mesometrial triangle (MT) including DB at gd6 or DB ± the transient mural thickening called the mesometrial lymphoid aggregate of pregnancy (MLAp) (combined or independent as indicated in the results) and fetal placenta at gd10 were dissected, homogenized in buffer and immediately used for isolation of total RNA using the *Rneasy Mini Kit* (Qiagen) according to manufacturer's instructions. At least two independent dissections and RNA preparations were made for each group studied. A *First-strand cDNA Synthesis Kit* (Amersham Pharmacia Biotech) was used to generate cDNA, in a 33 µl mixture containing 2.5g total RNA, 11 µl bulk first-strand cDNA mix, 1µl DTT, 1µl primer Not-I-d $(T)_{18}$ diluted 1:25 in DEPC H_2O. The reaction was incubated first at 37°C for 1 h, then at 90°C for 5 min to inactivate the enzyme and denature the RNA:DNA strands. One µl of cDNA was then subjected to PCR in a 15 µl volume containing 11.555 µl dd H_2O, 1.5 µl 10x PCR Reaction Buffer (Sigma), 0.3 µl dNTP (Amersham Pharmacia Biotech), 0.045 µl Taq DNA polymerase (Sigma; 5u/µl) and 0.3 µl of the sense and anti-sense primer for each gene (10 pm/µl), as given in Table I. Cycle numbers, time and temperature used were as previously reported (30,46-48). PCR products were separated and sized on 1% agarose ethidium bromide gel.

Table I. Primers used for LY49 PCR

Sequence	Forward	Reverse	Amplimer (bp)
Ly49A	aagtctatggagtgtgatc	gcagactaagtccaatgg	590
Ly49B	ccgtcaagagtaccaggtc	Agcagattgagttcttccc	585
Ly49C	ctccaccagcatcactccg	caagaaacgaataaggatcaactc	908
Ly49D	gaaaaagctcgcctcagag	catatgacaatccaatccag	506
Ly49E	tagattgcagtccaggtgag	tctgtctccaagaggaagg	606
Ly49F	tgctggcagctcattgtgaa	gttcattgcctggcctagag	220
Ly49G1	cacgaaagacccatctcca	attgcaggaaacaaatgtgag	480
Ly49G2	tcctcacagcacacagg	gataagctttgggggacc	543
Ly49G3/4	aggaatcacttctcagtaga	attgcaggaaacaaatgtgag	756/948
Ly49H	aggcatccattctttctacc	actcttggtttcactgtccc	486
Ly49I	atcatgaggttgagtatcacc	gcaaagcgtgcccattcac	1122
Ly49J	aatcagtccatgtcagggta	ccctgaagacttatgaagat	240
Ly49Q	gtgctctatcccctggcatc	cctgtactacactctagagg	224
DAP12	ctggtgtactggctgggatt	tgcctctgtgtgttgaggtc	208

DBA lectin histochemistry. Virgin B6 uterus and gd6-15 implantation sites (B6, *scid/scid*, *nu/nu* and IL-15$^{-/-}$) were collected, fixed in 4% paraformaldehyde in PBS (0.05M, pH 7.4 (PFA)) for 3 h or in Bouin's fixative (Fisher Scientific) overnight, processed into paraffin, sectioned at 7 μm and mounted onto glass slides. Sections were deparaffinized, hydrated and subjected to DBA lectin histochemistry (2,49). In implantation sites, DBA reactivity is detected on plasma membranes of all uNK cell stages, uNK cell granules and some endothelial cells. Morphology distinguishes DBA$^+$ lymphocytes from endothelium. Tissues were examined by light microscopy and photographed using a Spot camera.

In situ hybridization for LY49B and LY49F. Tissues to be used for in situ hybridization were fixed in PFA, embedded by hand and cut as 5 μm sections. Slides were dewaxed in xylene and hydrated through graded alcohols into DEPC-treated PBS, refixed in PFA for 20 min, digested with proteinase K (20 min, 37°C). Antisense and sense RNA probes were prepared using the TOPO TA cloning kit (Invitrogen) and labeled with digoxigenin (DIG RNA Labeling Kit, Boehringer Mannheim), according to manufacturer's instructions. Sections were hybridized with probes 4 min at 95°C under coverslips, then incubated 6 h at 65°C. After washing, slides were incubated 1 h with alkaline phosphatase-conjugated anti-DIG antibody (1/500) in a blocking mixture (DIG Wash and Block Buffer Set, Roche). Color was developed with BCIP/NBT (Sigma).

RESULTS AND DISCUSSION

All uteri studied expressed DAP12 message. Virgin B6 uterus expressed LY49D, H, I, J and Q although no DBA lectin reactive cells were detected histologically (Fig 1a). LY49G2$^+$ lymphocytes have been reported in infant and virgin adult uteri from randombred mice using immunohistology (7). Virgin uteri from randombred *scid/scid* showed expression of LY49G2 but uteri from B6 and its related mutant

strains did not. Virgin uteri from RAG-2$^{-/-}$ and *nu/nu* expressed the broadest repertoires but they were not identical. RAG-2$^{-/-}$ lacked LY49F, G2 and Q while *nu/nu,* a strain known to have endogenously high NK cell function (13), lacked LY49G2, G3/4 and Q. Virgin uteri from IL-15$^{-/-}$ expressed the smallest repertoire, LY49B, J and Q, and IL-15$^{-/-}$ was the only strain to lack expression of the activating receptors LY49D and H. These data suggest some of the expression of LY49B, J and Q is from T cells. Further, the data suggest that expression of LY49D and H in the virgin uterus is restricted to uNK progenitor cells and that undefined activation molecules are present in the 15$^{-/-}$ uterus that signal via DAP12. Interestingly, the repertoires detected in the virgin uteri from randombred *scid/scid* and B6-RAG-2$^{-/-}$ were dissimilar. The differences between randombred *scid/scid* and B6-RAG-2$^{-/-}$ may be due to dissimilarity in strain backgrounds. Both strains expressed larger repertoires than B6. In sum (Fig. 2), these data suggest that, except in IL-15$^{-/-}$, uNK precursor cells are present in virgin uterus, they are regulated through balanced expression of activating and inhibiting signals and they express larger NKR repertoires when T cell regulation is removed.

By gd6, DBA-lectin reactive cells are present in mesometrial decidua in uNK+ strains, indicating modification of terminal sugars on uNK cell membranes to N-acetyl-galactosamine (Fig. 1b). Splenic NK cells from virgin mice, like NK cells in virgin uterus, do not display this carbohydrate (not shown). In B6 mice, all 14 LY49 genes were expressed (Fig. 2 and Fig. 3), despite relatively low numbers of uNK cells at this gd and lack of the morphologically most mature uNK cell subset (Fig 1b). Thus, pregnancy induced expression of inhibitory receptors only (LY49A, B, C, E, F, G1, G2, G3/4) and early uNK cells (gd6) display the full repertoire, even in homozygously mated B6 females seeing no foreign paternal MHC antigens. Gd6 *scid/scid* also gained in repertoire expression but remained void for LY49J, again supporting the idea of significant LY49J expression by T cells in the uteri studied. The overall data (Fig. 2) indicate that LY49 gene expression does not correlate to the four morphologically

recognized uNK cell subsets.

At gd10 (Fig 1c-h), when all 4 uNK cell subtypes are found (Fig 1c), LY49 genes expressed in DB of B6 and in *scid/scid* of shipment #1 matched those detected at gd6 (Fig. 2). This confirms work in lymphoid tissue suggesting that once LY49 receptor expression occurs, it is sustained (34-37). Uteri from *scid/scid* of shipment #1 at gd10 remained negative for LY49J expression. Samples were also available at gd10 from *nu/nu* and IL-$15^{-/-}$. Both the DB and MLAp from *nu/nu* expressed the entire repertoire, showing gain over virgin uteri, in expression of LY49G2, G3/4 and Q. IL-$15^{-/-}$ mice are among the strains that completely lack NK and uNK cells (Fig. 1f). Implantation sites in IL-$15^{-/-}$ resemble those reported for Tgε26 and RAG-$2^{-/-}$ /$\gamma c^{-/-}$ and display lack of MLAp development, failure of spiral artery dilation and decidual hypocellularity with edema (50). For IL-$15^{-/-}$, only DB could be dissected and pregnancy induced expression of LY49C, E, G1 and G2 in non uNK cells of the DB. LY49A, D, F, G3/4, H and I were not expressed in midgestation or virgin IL-$15^{-/-}$ uteri, indicating that these receptors, which include both activating and inhibiting receptors, must be expressed exclusively in the uterus by uNK cells..

In B6 and *nu/nu*, the full LY49 repertoire and DAP12 were expressed in the fetal part of the placenta at gd10 (Fig. 2). UNK cells are much less frequent in this region (Fig. 1d) where, by ultrastructure, they appear to interact with labyrinthine trophoblast (51). UNK cells localized within the fetal placenta are DBA lectin + (Fig 1d). Our findings concerning the repertoire in uNK cells associated with trophoblast parallel data from human decidual $CD56^{+}$ cells that document use of the full KIR repertoire and no induction of novel KIR. Skewing of the repertoire was found in human uNK cells but this could not be assessed in mice by the techniques we used (6,21,22). LY49 expression in placentae from IL-$15^{-/-}$ differed from that of DB by absence of only a single receptor, LY49C. Unexpectedly, the initial pair of samples from placentae of gd10 *scid/scid* (shipment #1) showed DAP12 expression but no

LY49 expression, which suggests that undefined molecules in pregnant uteri may utilize DAP12. A second group of randombred, predominantly H-2^b *scid/scid* (shipment #2) was purchased for study. Placentae (gd10) from these expressed all LY49 molecules analysed except G3/4 and J, a pattern identical to that expressed in DB and MLAp of the same animals. Because dissection into fetal and maternal tissue is imprecise (52), DBA lectin histochemistry was used between gd8-15 to address whether *scid/scid* placentae were unique. In comparison to B6 or *nu/nu, scid/scid* placentae (shipments #1 and #2) differed by the rarity with which DBA lectin + cells were present (compare Fig 1g to 1d). It seems plausible that recognition of trophoblast cells expressing developmental and, for outbred matings, paternal antigens could activate antigen specific cells and lead to changes in cell addressins or chemokine production that would attract uNK cells into placental regions (53,54). Thus, B cells and/or T cells that differentiate independently from the thymus, may contribute to the signaling pathways recruiting maternal uNK cells into the fetal compartment. Careful re-examination of SCID placentae will be necessary to determine whether the absence of uNK cells from that domain alters properties of microvessels or intravascular trophoblast cells, including depth of trophoblast invasion towards the maternal spiral arteries. Interpretation of these data as an apparent weakness in or inability of *scid/scid* placentae to recruit uNK cells is consistent with an earlier report that numbers of uNK cells in DB and MLAp of *scid/scid* are higher than in wildtype mice (1).

To assess whether the LY49 repertoire of uNK cells found within the vasculature differed from that of uNK cells in tissue, additional gd10 B6 mice were perfused extensively at euthanasia to remove intravascular cells (Fig. 1e). In the maternal DB region of the placenta, where high numbers of uNK cells are found (compare Fig 1c to 1d), there was no loss of gene expression through removal of intravascular cells. In contrast, as shown in Fig 2 and 3, intravascular cells removed from the fetal region of the placenta were the only cells in the tissue to express LY49-A, C, F and I and must represent

a specialized subset. Intraluminal murine uNK cells are large mature cells and 3 of the 4 receptors they express were not expressed in virgin B6 uteri. Endothelium does not line the vessels in which the intraluminal uNK cell subset(s) was demonstrated; rather, these vessels are lined in mouse and human by fetal trophoblast (55-57). This, plus the absence of LY49C from the placentae but not DB of IL-15$^{-/-}$ (both tissues lacked LY49A, F and I) suggests a single or a heterogenous subset of uNK cells expressing only the inhibitory receptors LY49A, C, F and I is carefully positioned in B6 mice to interact with trophoblast cells. Intraluminal trophoblast is not found in the spiral arteries of mice in the first half of gestation but does extend into these arteries early during human pregnancy (56,57).

Mouse uNK cells do not exist as cell lines and adequate conditions for their culture have not been reported. Their large, terminally differentiated size limits success in isolating them as a purified cell population and accounts for our study of dissected tissues enriched for the cell type. To support our interpretation, that uNK cells expressed the LY49 molecules we detected, a final series of in situ hybridization studies was undertaken between gd6 to 14 using probes to LY49B and LY49F and implantation sites from B6, *scid/scid* and IL-15$^{-/-}$. In B6, the LY49B+ and LY49F+ cells had frequencies, sizes and microdomain localizations of uNK cells, including strong detection within the myometrium (Fig. 4A,C,D). In *scid/scid* (shipment #2), most reactivity at gd6 was in the mesometrial decidua (not shown). Later, LY49+ cells in *scid/scid* were intravascular and found within DB and MLAp (Fig. 4E). For LY49B in IL-15$^{-/-}$ at gd6, very little signal was observed (Fig. 4B compared to Fig. 4A). The signal came from rare, lymphoid cells in the DB and myometrium. Because NK cells do not differentiate in IL-15$^{-/-}$ and no DBA lectin + cells were present (Fig.1f), it was concluded that the gd6 LY49+ cells in IL-15$^{-/-}$ were probably T cells.

This study of NKR supports previous phenotypic and genetic analyses that concluded the large granulated lymphocytes found in decidualizing uteri are NK cells. UNK cells do not appear to be an

unusual subset with restricted NKR, despite their unusually large diameters/volumes and exceptionally heavy cytoplasmic granulation. No developmental pattern of LY49 gene expression was seen in uNK cells. As soon as immature, DBA-lectin+ cells were found in normal B6 mice, all receptors were detected. Future studies will be necessary to determine if the cells expressing LY49D, H, I, J and Q in the virgin B6 uterus give rise to progeny that express the entire repertoire or if the cells expressing LY49A, B, C, E, F, G1, G2 and G3/4 represent a separate population of NK cells that homes to the uterus in response to ovarian steroids and uterine decidualization. There was no support for localization of distinct NK subsets within the major placental domains of maternal decidua or fetal trophoblast or within the uterine wall where most uNK cell proliferation occurs. However, cells expressing LY49 A, C, F and I were only present in vessels of the placenta lined by endovascular trophoblast, and these cells maybe a subset with unique functions. A barrier to migration of maternal NK cells into fetal placental regions has also been documented in *scid/scid* dams lacking T and B cells but not in *nu/nu* dams lacking thymus-derived T cells. This study highlights complex interrelationships between innate and antigen specific lymphocytes in the uterus, trophoblast, endothelial cells, cell surface glycosylation, cell homing and steroid hormones and demonstrates the value of in vivo study of the maternal-fetal interface during normal pregnancy.

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ACKNOWLEDGEMENTS

We thank Mr. Gordon Black for assistance with histology, Mr. Jianhong Zhang for assistance with illustrations, Dr. Michael Caliguiri for transfer of IL-15$^{-/-}$ breeding pairs and staff of the OMAFRA-OVC Isolation Unit for their dedicated care of our immune deficient mouse colony.

FIGURE LEGENDS

Figure 1. Photomicrograph of DBA lectin stained uterine sections with the mesometrial side of the uterus at the top of each image. A) From a virgin B6 uterus showing absence of lectin reactive cells. Endometrium (END), luminal epithelium (LE) and lumen (L) are labeled. B) From a fully decidualized B6 implantation site at gd6. Mesometrial triangle (MT) and embryo within the embryonic crypt (E) are labeled. Insert is detail of a morphologically immature DBA lectin+ uNK cell (arrowhead). C) B6 implantation site at gd10 with numerous DBA lectin + uNK cells (arrows) within decidua basalis (DB). Decidual vessels are marked V. Insert at top left gives detail of morphologically most mature DBA lectin + uNK cell (arrowhead). D) B6 decidua (DB)-trophoblast (PL) interface within the placenta at gd10. Insert shows uNK cell detail inside a blood vessel (arrowhead) in the labyrinthine trophoblast region). E) B6 (gd10) decidua basalis showing numerous uNK cells of differing sizes. Many of the lymphocytes are within blood vessels (arrows). F) IL-15$^{-/-}$ decidua (DB) at gd10. This tissue lacks DBA lectin+ uNK cells. Unmodified spiral arteries are marked SA. G) *scid/scid* decidua (DB)-trophoblast (PL) interface within the placenta at gd10. Numerous DBA lectin+ uNK cells are present in DB but are rarely found in the fetal trophoblast area of the placenta. H) is a line diagram of the normal gd10 implantation site indicating the transient mural structure rich in proliferating uNK cells, called the mesometrial lymphoid aggregate of pregnancy (MLAp), decidua basalis (DB), trophoblast cells of the placenta (PL) and the relative position of the fetus and its other membranes (Embryonic cavity). Locations of images C-G are indicated as boxes.

Figure 2. Grid indicating the 14 LY49 genes and DAP12 signalling molecule investigated and summarizing the RT-PCR findings in various tissues from virgin, gestation day (GD) 6 and GD 10 uteri from mice of different genotypes. For GD 6, mesometrial triangle was dissected which is largely decidua basalis. At GD10 for B6, *scid/scid* (SCID) #2 (second shipment of this randombred genotype)

and *nu/nu* (Nude), three tissues were assessed, the fetal part of the placenta (PL), the maternal part of the placenta called decidual basalis (DB) and the mesometrial lymphoid aggregate of pregnancy (MLAp), a transient uterine wall structure. For SCID #1 (first shipment of this randombred genotype) and the perfused (Perf) B6 (2 independent samples), the MLAp and DB were combined. For the IL-15$^{-/-}$, no MLAp develops and only decidua basalis and placenta were available for study. Black boxes indicate amplification of a band of appropriate size. Blanks are left where no signal was detected.

Figure 3. Example of RT- PCR products for LY49B (Lanes 1-5) and LY49A (Lanes 6-10) showing removal of LY49A but not LY49B from placental regions by perfusion. B6 gd6 mesometrial triangle (Lanes 1&6); B6 gd10 decidua basalis (Lanes 2&7); B6 gd10 placenta (Lanes 3&8); B6 gd10 DB after perfusion (Lanes 4&9); B6 gd10 placenta after perfusion (Lanes 5&10); negative amplified water controls in lanes marked (-); 100 bp DNA ladder in lane marked bp.

Figure 4. Examples of in situ hybridization studies. Mesometrial aspect of the uterus is to the top of each panel. A) B6 gd6 and B) IL-15$^{-/-}$ gd6 hybridized with antisense LY49B. Reactive cells are distributed mesometrially, primarily in the decidua basalis of B6 mice. Myometrium (M), lateral decidua (LD), antimesometrial decidua (AMD) and embryonic crypt (EC) are indicated. Insert shows relative size and frequency of LY49B+ cells (arrows) in B6. In comparison, only very rare cells hybridized with LY49B in IL-15$^{-/-}$. C-E depict studies with gd12 B6 hybridized with antisense LY49F in C; antisense LY49B in D and sense LY49F in E. Strong hybridization was observed to similar cells using each antisense probe in the uterine wall (MLAp; insert in C, arrow) and in the decidua basaltis (DB, insert in D, arrow). A blood vessel (BV), surrounded by LY49+ cells is prominent in both C and D. Panel F is antisense LY49B hybridization on gd10 IL-15$^{-/-}$. No signal was detected above background. Panel G) is LY49B antisense hybridization on gd10 SCID. Reactive cells (arrows) were prominent in uterine wall and DB. Insert shows intravascular LY49B+ cell at gd14.

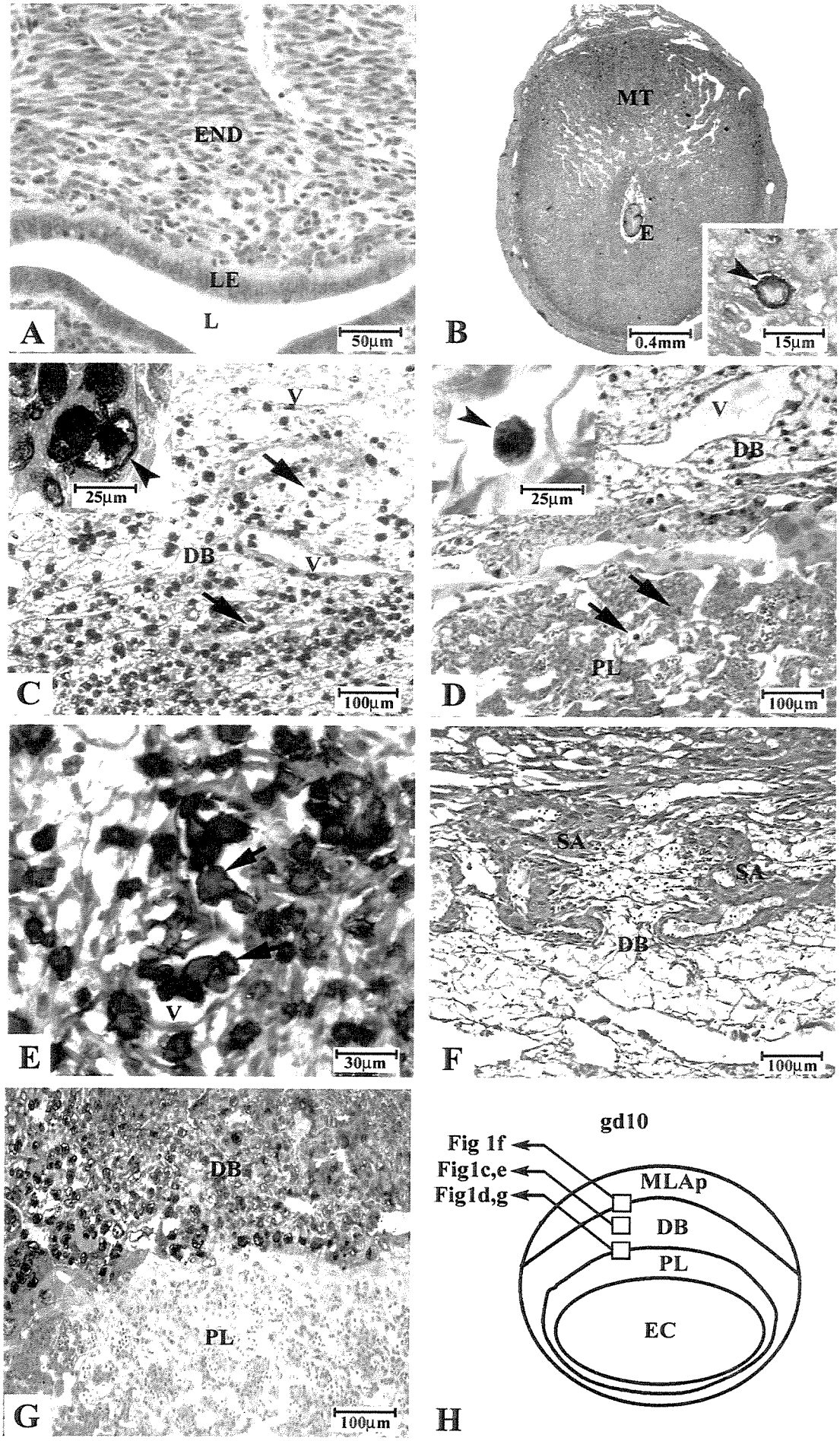
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Figure 1

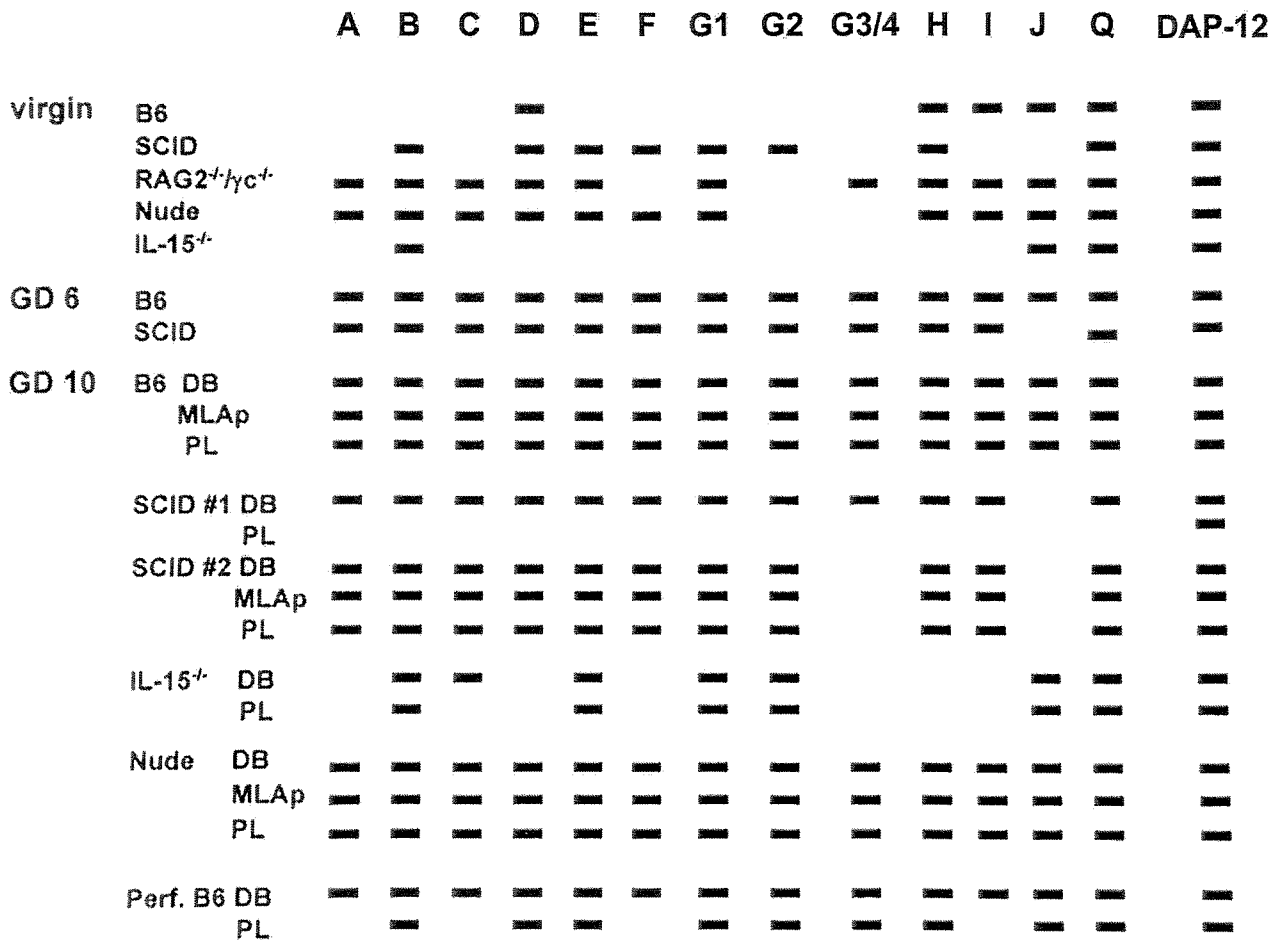
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Figure 2

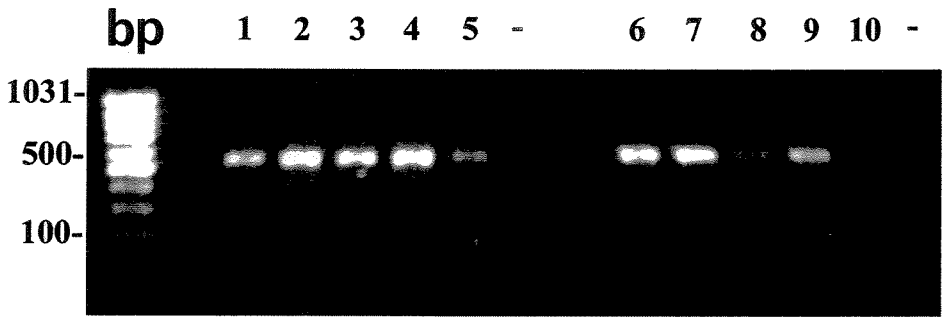
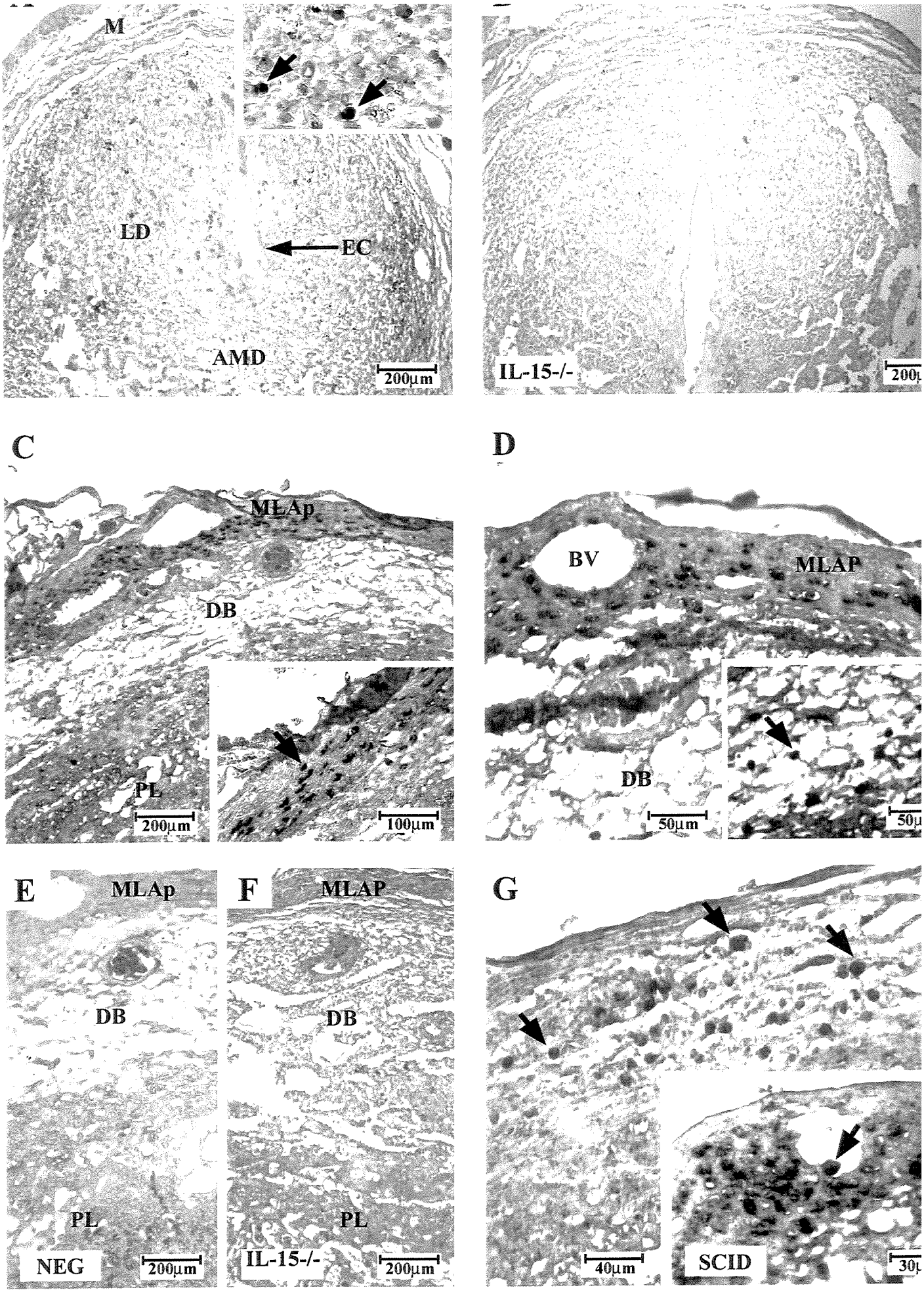


Figure 3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CAPÍTULO- V

CONCLUSÕES

1- A lectina *Dolichos biflorus* (DBA) reage de forma altamente seletiva com glicoconjugados expressos exclusivamente pelas células NK do ambiente uterino, com sensibilidade e especificidade comparável a um imunomarcador. A expressão do glicoconjugado de superfície reativo à lectina DBA pelas células NKu, parece ser um fator determinante do comprometimento destas células no ambiente uterino.

2- Através da lectina DBA estabeleceram-se novos parâmetros morfológicos para as células NKu, permitindo identificar 4 subtipos de maturação no ambiente uterino, além de, permitir uma avaliação precisa da localização e incidências destas formas celulares ao longo da gestação.

3- As células NKu não expressam receptores de estrógeno ERα ou ERβ, não sendo portanto a indução direta pelo hormônio estrógeno, o fator determinante para a proliferação, diferenciação e atividades funcionais destas células no útero durante a gestação.

4- As células NKu expressam um grande repertório dos genes de receptores LY49 comumente encontrados nas NKc e que independem também do estágio de maturação, o que sugere não serem estes genes expressos de forma distinta entre as NKc e NKu, porém, a prenhez induz a expressão de receptores Ly49 inibitórios nas células NK.

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Authors:
Manuscript No: PL-02-90079R1

Dear Dr. Yamada

Thank you for returning the above manuscript which is now accepted for press. It will be forwarded to the publishers and you will receive proofs from them for your approval.

Yours sincerely

Leslie Myatt
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ANEXO 1

Editorial Manager(tm) PLAC
Manuscript Draft

Manuscript Number: PL-02-90056R1

Title: Functional Analysis of Murine Natural Killer Cells Genetically Devoid of Estrogen Receptors

Article Type: Original Article

Keywords: mouse pregnancy, lymphocyte differentiation, lymp, ocyte homing, decidual spiral artery modification

Corresponding Author: Dr. B. Anne Croy Ontario Veterinary College

Other Authors: Angela M Borzychowski, Sirirak Chantakru, Kanwal Minhas, Valdemar A Paffaro, Jr., Aureo T Yamada, Hung He, Kenneth S Korach

ANEXO 2

Subject: LY49 MS
Date: Tue, 26 Nov 2002 17:09:21 -0500
From: "Anne B. Croy" <acroy@ovc.uoguelph.ca>
Organization: OVC
To: paffaro@ccshst09.cs.uoguelph.ca, junior <paffaroj@unicamp.br>, Yamada@ccshst09.cs.uoguelph.ca, "Áureo T." <yamadat@unicamp.br>

Just to let you know that the JI number is 02-4347. The submission date will be Nov 26 officially. Anne

ANEXO 3