National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le drcit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

CYTOKINES IN PLACENTAL DEVELOPMENT: TNF-α INDUCES APOPTOSIS IN CULTURED HUMAN TERM PLACENTAL TROPHOBLASTS

BY

© SUI-JANE YUI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

IN

IMMUNOLOGY

DEPARTMENT OF MEDICAL SCIENCES

EDMONTON, ALBERTA

FALL 1994

ISBN 0-315-95294-6

Name Sui-Jane Yui

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

Immunology
SUBJECT TERM

0982
SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION
General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523

Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES
American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES
Biogeochemistry 0425
Geochemistry 0996

Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES
Pure Sciences
Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences
Applied Mechanics 0346
Computer Science 0984

Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY
General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: **SUI-JANE YUI**

TITLE OF THESIS: **CYTOKINES IN PLACENTAL DEVELOPMENT: TNF-α INDUCES APOPTOSIS IN CULTURED HUMAN TERM PLACENTAL TROPHOBLASTS**

DEGREE: **DOCTOR OF PHILOSOPHY**

YEAR THIS DEGREE GRANTED: **FALL 1994**

Sui-Jane Yui

5th October 1994

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **CYTOKINES IN PLACENTAL DEVELOPMENT: TNF-α INDUCES APOPTOSIS IN CULTURED HUMAN TERM PLACENTAL TROPHOBLASTS** submitted by **SUI-JANE YUI** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **IMMUNOLOGY.**

Dr. Larry J. Guilbert (Supervisor)

Dr. Donald W. Morrish

Dr. Timothy R. Mosmann

Dr. David M. Olson

Dr. Joan S. Hunt (External Reviewer)

September 15, 1994

ABSTRACT

Lymphohematopoietic cytokines present at the maternal fetal interface have a major impact in affecting pregnancy outcome. In this study, I investigated the effects of tumor necrosis factor-α (TNF-α) on the survival, differentiation and function of cytotrophoblasts (CTs), progenitor cells which give rise to the multinucleated syncytiotrophoblast (ST) of the placental villi, a terminally differentiated tissue which forms a barrier at the maternal-fetal interface.

Purification of CTs from term placental tissues was achieved by elimination of CD9 positive mesenchymal cells using glass bead columns. In culture, these column-purified cells formed a monolayer of mononucleated CTs with greater than 99% purity and survived for 2 weeks without loss of viability. Proliferation was barely detectable in trophoblast cultures and partial syncytialization occurred in the presence of EGF.

The addition of TNF-α was cytotoxic to the cytotrophoblast monolayer as monitored by a reduction in MTT cleavage and DNA content. Interferon-gamma (IFN-γ) at 100 U/ml had no adverse effects on the trophoblasts but enhanced the action of TNF-α when the latter was used at low concentrations. Death occurred by a process known as apoptosis, as demonstrated by an increased level of DNA nicking and fragmentation into uniform nucleosome-sized oligomers. Apoptosis was also detected in syncytium exposed to high concentrations of TNF-α and IFN-γ.

With a combination of receptor-specific monoclonal antibodies and TNF-α mutants, it was found that TNF-α cytotoxicity in trophoblasts was transmitted primarily through the p55 TNF-Rs. The post-receptor signaling

components did not appear to involve nitric oxide or reactive oxygen intermediates, but sphingomyelinase mimicked the toxic effects of TNF-α, suggesting a ceramide-dependent pathway.

TNF-α induced cell death could be modulated by culture components. Protein and RNA synthesis inhibitors synergized with TNF-α and IFN-γ to enhance the cytotoxic effects whereas dexamethasone and EGF effectively prevented death, implying trophoblast survival rests on a balance between opposing sets of cytokines and hormones. In conclusion, I propose that TNF-α regulates placental development by selectively inducing trophoblast death via apoptosis, but aberrant expression of TNF-α could result in the premature depletion of progenitor CTs which in turn leads to small placenta, fragile ST barrier and intrauterine growth retardation.

ACKNOWLEDGMENTS

The completion of this thesis project would not have been possible without the contribution from the following people. I would like to take this opportunity to express my deepest thanks and gratitude to them.

I would like to thank my late supervisor, Dr. Tom Wegmann, for remembering my interests in human reproductive immunology and propelling me to take up this project once it got going. My thanks also go to Dr. Larry Guilbert, my present supervisor, for providing me a friendly and encouraging environment to work in, and for his dedicated and excellent supervision; to Dr. Tim Mosmann, for his critical reading of the thesis and for taking time in his busy schedule to work with me in interpretation of the experimental data; to Dr. Don Morrish, for encouragement and continual interest in this project. Last, but not the least, I would like to thank Dr. Maria Garcia-Lloret, for the time and effort she spent in guiding me throughout the steps of this project; from the planning of experiments to discussions of results and future experiments, and above all, for being there whenever advice is desperately needed.

I would like to thank Amanda Brown for teaching me the techniques of running the trophoblast columns when I first started this project and for providing the reagents to purify the cells; to Mr. Mike Wride for teaching me the TUNEL technique; to Dr. Robert Hardin in the Department of Animal Science and Mr. Jason Acker for helping with the statistical analysis; to Bonnie Lowen, Anne Smith for their excellent technical assistance; to Heather Ferguson for her excellent secretarial help and to the other students and technicians in the labs of Drs. Guilbert, Janowska and McGann for their freindships and assistance.

I would also like to thank the nurses at the caseroom of the University of Alberta for supplying me with the placentas and also the technicians at the Pathology Department of the University of Alberta Hospital for preparing frozen sections.

I would like to thank the brothers and sisters in Christ in the Edmonton Chinese Christian Fellowship (ECCF) and South Edmonton Alliance Church whom I met during these five years. Your prayers carry me through all these years! Thank you all for helping me to grow spiritually and teaching me to look beyond the present and to invest in the everlasting.

Above all, I thank God for giving me wonderful parents who fully support the path I took, and for this precious and memorable experience to be able to pursue graduate studies under the guidance of so many talented people.

Throughout my graduate studies, I am supported by a full-time studentship from the Alberta Heritage Foundation of Medical Research for which I am extremely grateful.

TABLE OF CONTENTS

CHAPTER **PAGE**

I. Introduction

A. Prologue 1

B. Structure and Development of the Placental Villi 2

- *Trophoblasts* 6

- *Basement membrane* 8

- *Villous stroma* 9

C. Cytokines in Placental villi 14

- *Interleukins* 15

- *Interferons* 20

- *Epidermal growth factor* 24

- *Transforming growth factor-β* 26

- *Colony stimulating factors* 27

- *Tumor necrosis factor* 34

II. Hypothesis, Rationale and Project

A. Hypothesis 41

B. Rationale 42

C. Experimental Approach 44

III. Materials and Methods

A. Materials and Methods (General)

- *Isolation of placental cells from term placenta* 46

- *Purification of placental cells via negative selection* 47

- *Culturing of column-purified cells* 47

- *Cytokines added to cultures* 48

- *MTT Assay* 48

- *Assay for DNA content* 48
- *Detection of apoptotic cells using TUNEL* 49
- *Statistical Analysis* 50

B. Materials and Methods Specific for Chapter IV

- *Preparation of tissue sections from term placenta* 50
- *Preparation of placental macrophages* 50
- *Preparation of placental fibroblasts* 51
- *Flow cytometric analysis of placental cells* 51
- *Immunohistochemical staining of cultured placental cells* 52
- *Immunofluorescent staining of cultured placental cells* 53
- *Measurement of hCG production* 53
- *Assay for protein content* 54
- *Proliferation assay for trophoblasts* 54

C. Materials and Methods Specific for Chapter V

- *Flow cytometric analysis of DNA content using propidium iodide* 54
- *DNA fragmentation assay* 55
- *Combined TUNEL and desmoplakin staining* 55

D. Materials and Methods Specific for Chapter VI

- *Anti-TNF-receptor antibodies* 56
- *Receptor-type specific TNF-α mutants* 56
- *Cell culture in arginine deficient medium* 57
- *Inhibitors of nitric oxide production* 57
- *Inhibitors of reactive oxygen intermediates (ROIs)* 58
- *Measurement of ROIs by fluorescence* 58
- *Reagents for sphingomyelin pathway* 58
- *Inhibitors for RNA and protein synthesis* 59

E. Materials Specific for Chapter VII

- *Reagents* 59

IV. Purification of human term placental trophoblasts by column elimination of CD9 expressing cells.

A. Introduction..... 60

B. Results

- Expression of CD9 in the stroma of term placental villi..... 63

- Removal of contaminating CD9 positive cells by passage through glass bead columns..... 63

- Comparison of pre- and post-column cells after one week in culture..... 64

- Synthesis of human chorionic gonadotropin (hCG) by column-purified trophoblasts..... 65

- Syncytialization of purified trophoblasts after a week in culture..... 66

- Purity and viability of long term trophoblast cultures..... 66

C. Summary of results..... 67

D. Tables and figures..... 68

V. TNF-α induces apoptosis in primary human placental cytotrophoblasts

A. Introduction..... 79

B. Results

- TNF-α alone and in combination with IFN-γ disrupts cytotrophoblast monolayers..... 82

- The disruptive effect of TNF-α on trophoblasts is concentration and time dependent..... 82

- Trophoblasts cultured in TNF-α and IFN-γ undergo apoptotic death..... 84

- Susceptibility of EGF-induced syncytium to the cytotoxic effects of TNF-α and IFN-γ..... 86

C. Summary of results..... 87

D. Tables and figures..... 88

VI. Mechanisms associated with TNF-α induced cytotoxicity in cultured trophoblasts.

A. Introduction 99

B. Results

- Involvement of the p55 and p75 TNF receptors in signal transduction of TNF-α mediated cytotoxicity 105

- Nitric oxide is not involved in TNF-α-mediated cytotoxicity of trophoblasts 106

- Reactive oxygen intermediates are not involved in TNF-α-induced cytotoxicity of trophoblasts 107

- Addition of sphingomyelinase mimics the cytotoxic effects of TNF-α on trophoblasts 107

- Inhibitors of RNA and protein synthesis enhance cell death 108

C. Summary of results 108

D. Tables and figures 110

VII. Antagonism of TNF-α induced cytotoxicity by EGF and glucocorticoids

A. Introduction 121

B. Results

- Dexamethasone is effective in inhibiting TNF-α induced cytotoxicity 123

- EGF-treated cultures demonstrate increased resistance to cytokine-induced cytotoxicity 123

- Dexamethasone and EGF prevent cell death by inhibiting apoptosis 124

C. Summary of results 124

D. Table and figures 125

VIII. Discussion

A. Introduction..... 129

B. Development of an *in vitro* culture model to study TNF-α effects on placental trophoblasts..... 130

C. TNF-α induces apoptosis in primary human placental cyto-trophoblasts..... 135

D. TNF-α mediates cytotoxicity in cultured cytotrophoblasts via p55 TNF receptors..... 138

E. Antagonism of TNF-α induced cytotoxicity by EGF and glucocorticoids.. 143

F. Proposal of a model for TNF-α activity in placental development..... 145

G. Future Directions..... 150

IX. Bibliography..... 154

LIST OF TABLES.

Tables	Description	Page
1.1	Localization of cytokines and their corresponding receptors to cells of the placental villi	40
4.1	Comparison of cell yields and cell purity before and after column passage	68
4.2	Comparison of vimentin and CD9 positive cells in pre- and post-column cultures after two weeks	69
4.3	Viability, DNA and protein content of post-column cells cultured for two weeks	70
5.1	Correlation between MTT reductive capacity and DNA content of trophoblasts cultured with TNF-α and IFN-γ	88
5.2	Susceptibility of syncytium to TNF-α and IFN-γ treatment	89
6.1	Effects of htr-9 and utr-1 on TNF-α-induced cytotoxicity in cultured trophoblasts	110
6.2	Effects of arginine on the cytotoxicity of trophoblasts mediated by TNF-α and IFN-γ	111
6.3	Effects of NO inhibitors on the cytotoxicity of trophoblasts in the presence of TNF-α and IFN-γ	112
6.4	Generation of ROIs by trophoblasts treated with TNF-α and IFN-γ	113
6.5	Effect of ROI inhibitors on the cytotoxicity of trophoblasts stimulated by TNF-α and IFN-γ	114
6.6	Effects of cycloheximide and actinomycin D on trophoblasts cultured for 18 hours in the presence of TNF-α and IFN-γ	115

7.1 Effect of EGF on TNF-α-induced cytotoxicity of cultured trophoblasts..... 125

LIST OF FIGURES

Figure	Description	Page
1.1	Diagram of anchoring and floating villi at the fetal-maternal interface.....	5
4.1	Localization of CD9 in placental villous stroma by immunoperoxidase staining with anti-CD9 antibody 50H.19.....	71
4.2	Flow cytometric analysis profiles of immunofluorescence-stained placental cells before and after column passage.....	72-73
4.3	Immunocytochemical staining of cytokeratin on post-column trophoblasts.....	74
4.4	Giemsa staining of pre- and post-column cultures.....	75
4.5	Production of hCG by trophoblast cultures.....	76
4.6	Anti-desmoplakin staining of column-purified trophoblasts with and without EGF.....	77
4.7	Comparison of mono- and multi-nucleated cells in untreated and EGF-treated cultures.....	78
5.1	TNF-α alone and in combination with IFN-γ disrupts trophoblast monolayers.....	90
5.2	Effects of TNF-α on trophoblasts as a function of concentration and time.....	91-92
5.3	Effects of transient TNF-α exposure on cell loss from trophoblast cultures.....	93
5.4	Comparison of numbers of non-adherent cells in trophoblast cultures with or without TNF-α and IFN-γ treatment.....	94
5.5	Early morphological changes induced by TNF-α and IFN-γ in trophoblast cultures demonstrated by TUNEL.....	95

5.6 Flow cytometric analysis of DNA content in non-adherent cells..... 96

5.7 DNA fragmentation in cultured trophoblasts as visualized by gel electrophoresis..... 97

5.8 Demonstration of apoptosis in syncytium exposed to TNF-α and IFN-γ.. 98

6.1 Effect of htr-9 and utr-1 antibodies on the induction of cytotoxicity in trophoblasts..... 116

6.2 Cytotoxic activity of wild type and TNF-α mutants in trophoblasts..... 117

6.3 Inhibition of ROIs generation..... 118

6.4 Concentration dependency of sphingomyelinase in the induction of trophoblast cytotoxicity..... 119

6.5 Viability of placental trophoblast and fibroblasts as a function of the concentration of CHX and act D..... 120

7.1 Effect of dexamethasone on TNF-α/ IFN-γ-induced cytotoxicity of cultured trophoblasts..... 126

7.2 Effects of GM-CSF, CSF-1 and EGF on TNF-α/ IFN-γ-induced cytotoxicity of trophoblasts..... 127

7.3 Reduction in the numbers of apoptotic nuclei in cultures treated with dexamethasone and EGF..... 128

8.1 Hypothetical model for the function of TNF-α in placental development..... 152-153

ABBREVIATIONS

Act D	Actinomycin D
AIDS	Acquired immunodeficiency syndrome
AV	Anchoring villi
BHA	butylated hydroxyanisole
CHX	Cycloheximde
CSF(s)	Colony-stimulating factor(s)
CT(s)	Cytotrophoblast(s)
DCF-DA	Dichlorofluorescin-diacetate
DDW	Doubled-distilled water
Dex	Dexamethasone
DMEM	Dulbecco's modified eagle medium
EBV	Epstein Barr virus
EC	Endothelial cell
EGF	Epidermal growth factor
EM	Electron microscopy
ENDO	Endometrium
ER	Endoplasmic reticulum
FB	Fibroblasts
FCS	Fetal calf serum
FV	Floating villi
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hanks Buffered Salt Solution
hCG	Human chorionic gonadotropin
HIV	Human immunodeficiency virus

HLA	Human leukocyte antigens
hCG	Human chorionic gonadotropin
hPL	Human placental lactogen
hsp	Heat shock protein
IFN(s)	Interferon(s)
IMDM	Iscove's modified Dulbecco's medium
Ig	Immunoglobulin
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IUGR	Intrauterine growth retardation
K_d	Equilibrium constant
LAK	Lymphokine activated killer cells
LGL	Large granular lymphocytes
LPS	Lipopolysaccharide
LT	Lymphotoxin
Mϕ	Macrophage
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MY	Myometrium
NK	Natural killer cells
NAME	Nω-nitro-L-arginine methyl ester
NMMA	N^G-monomethyl-L-arginine monoacetate salt
NO	Nitric oxide
op	Osteopetrotic
PAI-2	Plaminogen activator inhibitor type 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-B-R	B-isoform of the platelet derived growth factor receptor

R(s)	Receptor(s)
RIA	Radio-immunoassay
RNIs	Reactive nitrogen intermediates
ROIs	Reactive oxygen intermediates
RT	Room temperature
S.D.	Standard deviation
SMase	Sphingomyelinase
ST	Syncytiotrophoblast
TdT	Terminal deoxynucleotidyl transferase
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TUNEL	TdT-mediated dUTP-biotin nick end labeling

CHAPTER I
INTRODUCTION

A. Prologue

In human pregnancy, the maintenance of the semi-allogeneic conceptus in the uterus of its mother has been viewed as a paradox in transplantation immunology. The success of this allograft is partly attributed to the placenta, an extra-embryonic organ that is situated strategically at the maternal-fetal interface to provide an immunological barrier and that is endowed with synthetic, secretory, transport and invasive capabilities essential for the maintenance of pregnancy. In addition, the human placenta undergoes dramatic structural reorganization and functional specialization during pregnancy in order to synchronize with the development of the embryonic/ fetal and maternal compartments (Ohlsson, 1989). The first part of this introduction is therefore to familiarize the readers with the microstructure of this dynamic tissue.

The importance of the placenta as an endocrine organ in the synthesis of steroid and peptide hormones has been well-studied. Recently however, a new group of polypeptide molecules, known as the cytokines, have been localized to the placenta where they establish a localized network that may play a significant role in many biological mechanisms controlling the reproductive processes. In the second part of the introduction, I therefore give a brief overview of those cytokines which have been reported to play a role in pregnancy. I chose to study tumor necrosis factor-α (TNF-α) because its abundance in the placenta and its temporally regulated expression during pregnancy suggest an important role in placental development and function.

Because TNF-α is one of the few cytokines known to have direct cytotoxic effects of cells, I postulate that TNF-α induce the death of trophoblasts, epithelial cells which line the apical surface of the placenta. This process of cell death could either be involved in regulating placental homeostasis or result in pathological manifestations within the placenta when TNF-α activity escapes normal control mechanisms.

B. Structure and Development of the Placental Villi

The intricate processes of implantation and placentation are critical to mammalian fetal development. In human reproduction, the fertilized embryo undergoes several cleavages to become a blastocyst surrounded by an outer layer of cells called trophoblasts. The term "trophoblast" was first introduced by Hubrecht in 1889 to describe a component of the hedgehog blastocyst which supplies nourishment to the fetus but does not contribute to forming the embryo proper (Boyd and Hamilton, 1970). During implantation, which in human occurs about one week after coitus, the trophoblast attaches to the maternal uterine wall and begins an invasive process which results in the penetration of maternal spiral arteries and the establishment of the hemochorial type of placentation where maternal blood flow comes into direct contact with fetally-derived trophoblasts (Aplin, 1991; Strickland and Richards, 1992). In the next few months, the trophoblasts differentiate along two distinct pathways, giving rise to the formation of the anchoring villi and the floating villi (Damsky, Fitzgerald and Fisher, 1992).

The anchoring villi are formed early in pregnancy to attach the embryo to the maternal uterine wall, which also undergoes physical and biochemical changes to form the decidualized endometrium in order to accommodate for

the demands of pregnancy. Trophoblasts within the anchoring villi, also known as extravillous trophoblasts, are organized into multilayered columns of non-polarized cells penetrating deep into the maternal endometrium (Damsky, Fitzgerald and Fisher, 1992). The extravillous trophoblasts acquire a migratory and invasive phenotype comparable to certain aspects of cancerous behavior and accordingly they also express many of the genes associated with proliferation, such as *myc* and *sis* (Ohlsson, 1989), as well as integrin receptors for interaction with the extracellular matrix (ECM). This "pseudo-malignant" process, referring to the invasiveness of the extravillous trophoblasts, occurs in two waves, one in the first trimester followed by another in the second trimester up to about 18 weeks' gestation (Aplin, 1991). The final result of the invasive process is the replacement of a collagenous matrix by a unique fibrinoid deposit which permits the expansion of arterial walls and reduces resistance of blood flow into the intervillous spaces. Aberrant regulation of gene expression for various growth factors and integrins in the control of trophoblast proliferation and invasion may result in placental diseases such as pre-eclampsia, choriocarcinomas and hyatidiform moles (Zhou et al., 1993).

In contrast to the anchoring villi, the floating villi do not contact the endometrium and exist as polarized tissue structures. Throughout placental development, the floating villi serve many important metabolic functions supporting fetal growth. These include gaseous exchanges, nutrient transfer, excretion, resorption, synthesis, secretion and the maternal-fetal barrier (Benirschke and Kaufmann, 1990). The size of the villi progressively decreases throughout pregnancy, measuring an average of 170 μm in diameter in the first trimester to 40 μm in the term placenta (Fox, 1978). Nonetheless, the same basic structure of the villi persists throughout gestation. Bathed in

maternal blood, the villi are covered by a continuous syncytium, which consists of a multi-nucleated syncytiotrophoblast (ST) devoid of distinct cellular boundaries. Beneath lies the cytotrophoblasts (CTs), the stem cells which proliferate (in the first and second trimesters but not in the third trimester) and fuse (in all three trimesters) to form the overlying syncytium. The trophoblasts are derived from the extra-embryonic trophectoderm and thus are of epithelial origin. The remaining cells in the floating villi, fibroblasts, fetal macrophages, endothelial cells, and a few mast cells, are of mesenchymal origin and they are located in the stromal core of the villi (Fox, 1978). The stroma is segregated from the trophoblasts by a basement membrane.

A schematic representation of the floating and anchoring villi is shown in Figure 1.1. Because this thesis project deals entirely with villous trophoblasts of the floating villi, extravillous trophoblasts of the anchoring villi will not be discussed here. For those readers interested in trophoblast differentiation and function along the extravillous lineage, a recent article by Damsky, Sutherland and Fisher (1993) provides a good overview in this area of research.

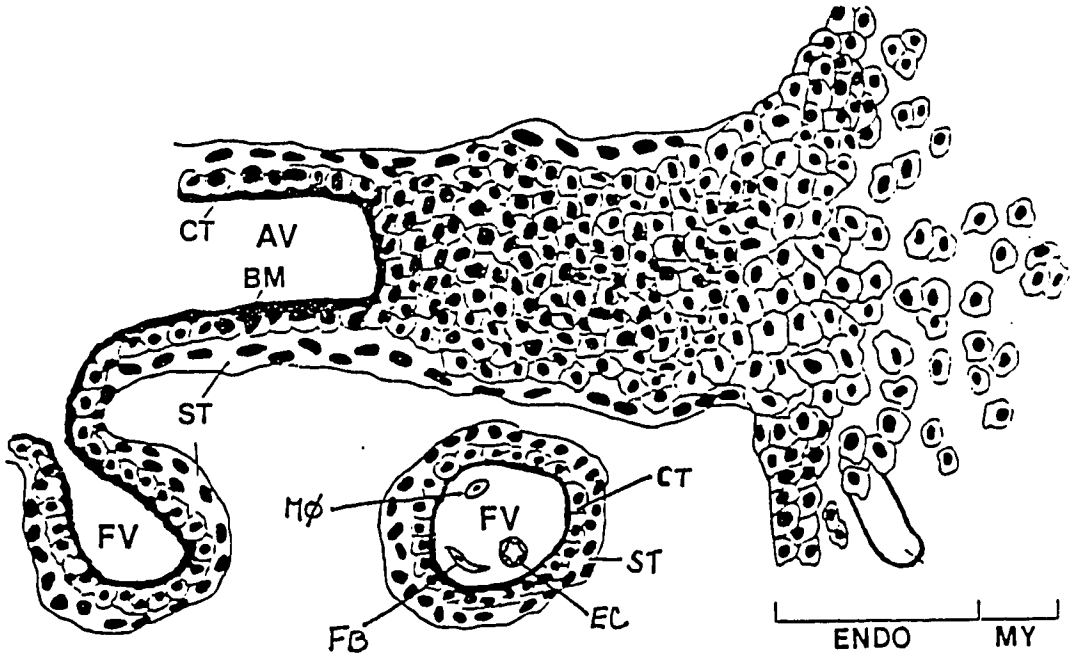


Figure 1.1. Diagram of the fetal-maternal interface showing floating villi (FV), an anchoring villus (AV) with an associated cell column, and the maternal uterine wall. In the FV, mononucleated CT stem cells, separated from the stromal core by a basement membrane (BM), fuse to form the overlying ST whose apical surface is bathed in maternal blood. Within the stromal core are fibroblasts (FB), macrophages (Mϕ) and fetal endothelial cells (EC). In the AV, extravillous trophoblasts proliferate and form a cell column which penetrates the maternal endometrium (ENDO) and myometrium (MY), thus anchoring the placenta to the maternal uterine wall. *(Adapted from Damsky, Fitzgerald and Fisher, 1989).*

The trophoblasts

Villous trophoblasts are epithelial cells that line the surface of the floating villi. In the first trimester, the two layers of trophoblasts, an outer layer of syncytium and an inner layer of mononucleated CTs, can be easily distinguished. The latter are also known as Langhans' cells, named after their discovery by T.H. Langhan in 1882.

Both villous and extravillous trophoblasts trace their origins to a population of primitive mononucleated stem cells which are highly proliferative (Aplin, 1991). The signals and mechanisms which control the differentiation into these two separate lineages are at present unknown. As the primary function of villous CTs is to undergo cell division without functional specialization, they demonstrate features characteristic of undifferentiated cells which have a large nucleus, prominent nucleoli and numerous free ribosomes within the cytoplasm but with rather poorly developed endoplasmic reticulum (ER) and Golgi (Contractor et al., 1977). By term, CTs constitute 20-40% of the villi, and they are absent from the basal surface of the ST in areas known as epithelial plates (Benirschke and Kaufmann, 1990). When present, they assume a flattened morphology sandwiched between the basement membrane and the ST, and are visualized using periodic acid Schiff staining (Fox, 1978).

Under electron microscopy (EM), desmosomes are found at the junctions of the syncytium and CTs (Fox, 1978). It was first believed that in human the syncytium arises by endomitosis, a process analogous to that found in the formation of giant trophoblasts in rodents during which there is replication of nuclei without separation of cytoplasm (Cotte et al., 1980). However, autoradiographic studies using tritiated thymidine indicated that the

label was found in the CTs and not in the syncytium (Benirschke and Kaufmann, 1990). In addition Galton (1962) showed that the DNA content of CTs had more than the diploid amount of nuclei, suggestive of interphase mitosis. Further evidence of syncytium formed by fusion of CTs rather than by endomitosis is provided by EM. Here, one can visualize the remnants of CT membrane fragments containing desmosomes within the ST (Contractor et al., 1977). In addition, there is the presence of transitional CTs (Boyd and Hamilton, 1970; Contractor et al., 1977) which on one side apposes an undifferentiated CT and on the other side, the ST. These transitional CTs possess well-developed ER and their mitochondria have cisternae resembling those of the ST. With recent improvements in methods for isolating and culturing trophoblasts, it has been shown by time lapse cinematography that CTs spontaneously aggregate and fuse to form syncytium (Kliman et al., 1986). Although syncytium formation can occur in basal medium, the supplementation of growth factors such as epidermal growth factor (Morrish et al., 1987; Barnea et al., 1990), the use of defined medium favoring the growth of epithelial cells (Douglas and King, 1990) and the seeding of extracellular matrices (Kao et al., 1988) can accelerate the degree of syncytialization.

At term, the syncytium demonstrates a pattern of mosaic areas with different structures and histochemical attributes, in contrast to the earlier stages of pregnancy when it is homogeneous with evenly distributed nuclei and cellular organelles (Benirschke and Kaufmann, 1990). These specialized regions start to appear during the second trimester and although they lack definite demarcations due to continuity of the syncytial nature, they can be differentiated on the basis of their thickness, nuclei distribution and the nature of their ER.

The thinnest regions in the term placenta are known as epithelial plates or vasculosyncytial membranes and measure between 0.5-1 μm. They are often devoid of nuclei and organelles, and are closely apposed to a bulging fetal capillary to facilitate water, gaseous and glucose exchanges between maternal and fetal circulations. Situated between epithelial plates are protrusions where aged syncytial nuclei accumulate. These areas, known as syncytia knots, possess small condensed nuclei showing severe pyknosis (Martin and Spicer, 1973). Another type of multinucleated protrusions, known as syncytial sprouts, occasionally dislodge from the villi and enter the maternal circulation where they can be deported to as far as the maternal lungs (Hawes et al., 1994).

Two types of syncytium can be classified on the nature of their ER. The most common type possesses rough ER and has extensive microvilli at the surface which participate in active transfer, cellular metabolism, and the synthesis of peptide hormones like human chorionic gonadotropin (hCG; Morrish, Marusyk and Siy, 1987), human placental lactogen (hPL; Morrish, Marusyk and Bhardwaj, 1988), prolactin and hormones involved in the initiation of labor such as oxytocin and β-endorphin (Unnikumar et al., 1988). The other type of ST contains prevailing smooth ER specializing in the metabolism of steroid hormones, like progesterone and estrogen (Boyd and Hamilton, 1970).

The Basement Membrane

The basement membrane of the placental villi provides a supportive scaffold for the attachment of the overlying CTs or in the case of epithelial plates, the ST. Immunohistochemical staining indicates that type IV collagen and all the isoforms of laminin constitute the predominant constituents in the

first trimester basement membrane, whereas staining for fibronectin is comparatively weaker (Damsky, Fitzgerald & Fisher, 1992). This is supported by the observation that the integrin $\alpha_6\beta_4$, a putative laminin receptor, is expressed primarily at the basal surface of CTs which are lying above the basement membrane (Korhonen et al., 1991; Damsky, Fitzgerald & Fisher, 1992). In first trimester villi, all stromal cells synthesize these basement membrane proteins, with endothelial cells being the most active (Autio-Harmainen, 1991). Although the thickness of the membrane increases throughout pregnancy, so far there has been no report of changes in its composition. However, it is likely that other matrix proteins may gain prominence as $\alpha_3\beta_1$, receptors for fibronectin and collagen, showed increased staining in term placenta (Korhonen et al., 1991). The regulation of integrin expression is more often studied in extravillous than in villous trophoblasts because the former interact with the maternal endometrium and integrins are well known for their role in cell-cell interaction. However the expression of integrin receptors in the villous trophoblasts and their corresponding ligands on the basement membrane has been implicated in the lateral movement of CTs along the basement membrane to sites where regeneration and repair of the ST are required since cellular motility involves alterations in integrin expression (Hynes and Lander, 1992).

The Villous Stroma

The villous stroma is a meshwork of reticular and collagen fibrils embedding undifferentiated mesenchymal cells, reticular cells, macrophages, endothelial cells, fibroblasts and a sprinkling of mast cells (Kaufmann et al., 1977). In accompaniment with the maturation of the trophoblasts, dynamic structural changes also occur in the villous stroma. In early pregnancy,

undifferentiated mesenchymal cells constitute the dominant cell type in the stroma; they are considered to be the stem cells from which other stromal cells including Hofbauer cells, fibroblasts and endothelial cells are derived (Dempsey, 1972). These progenitor cells are visible until the mid-trimester of gestation and are typically located in the stroma devoid of collagen fibres.

As pregnancy advances, these mesenchymal cells gradually increase their size, become more polygonal, and develop sail-like cytoplasmic processes with many branches. These differentiated cells are known as reticular cells and in one report, are claimed to be the precursors to fibroblasts and Hofbauer cells (Kaufmann et al., 1977). Fibroblasts are fixed connective tissue cells with well developed granular ER, numerous mitochondria and intracellular filaments but are devoid of sail-like processes characteristic of the reticular cells. They specialize in the synthesis of laminin and collagen, thus they contribute to the ground substance of the stroma and the framework of the basement membrane.

Placental macrophages are a major component of the villous stroma, constituting about 70% of the villous cell population at all stages of pregnancy (Wood, 1980). They display many features of mononuclear phagocytes including phagocytosis, positive staining for non-specific esterase and they express receptors for the Fc portion of immunoglobulin (Ig) and for the complement component C3. By using a panel of antibodies against monocyte/ macrophage markers, Goldstein et al. (1988) found that morphological variability was evident in placental macrophages. Apart from the round to oval-shaped Hofbauer cells which possess vacuolated cytoplasm and a small nucleus, other macrophages are spindle-shaped with long cytoplasmic processes, reminiscent of dendritic cells. On one hand, it is possible that the latter represents reticular cells. Alternatively, the variability of placental

stromal macrophages may attest to their different origins in that they may be the progeny of either fixed stromal cells of the villous core before fetal circulation is established or fetal bone marrow derived monocytes after the establishment of fetal circulation (Castellucci et al, 1987).

In addition to having receptors which facilitate the removal of complement and antigen-antibody complexes, placental macrophages have several other cell surface receptors that generate considerable interest. CD14, a 55 glycoprotein anchored in the membrane by a phosphatidyl-inositol tail, was originally viewed simply as a monocyte differentiation marker, but recently has been shown to bind lipopolysaccharide (LPS) complexed to serum binding proteins (Wright et al., 1990; Ziegler-Heitbrock and Ulevitch, 1993). Placental macrophages express CD14 in all three trimesters of pregnancy (Bulmer and Johnson, 1984; Goldstein et al., 1988). Soluble forms of CD14 have been described and they may play a role in the clearance of Gram-negative bacteria (Wright et al., 1990).

In specific immunity, macrophages play an important role in antigen presentation because they process extracellular antigens into peptides which form complexes with human leukocyte antigen (HLA) class II molecules on the cell surface for interaction with the appropriate T-cell receptors (TCRs). Placental macrophages express all three HLA class II antigens, DP, DQ and DR, on their surface. The expression of DP and DR progressively increases as gestation proceeds, whereas DQ is expressed exclusively in the third trimester and not in the first (Goldstein et al., 1988). This gestation-dependent expression may reflect an increase in functional maturity of placental macrophages as it has been reported that those derived from term placentas were able to stimulate the proliferation of maternal mononuclear leukocytes though not to the extent when cord blood cells were used as stimulators (Hunt,

King and Wood, 1984). A similar situation occurs in mice where placental macrophages and macrophage cell lines derived from mid-pregnancy have diminished ability to activate T-cell hybridomas as compared to that of spleen cells (Chang et al., 1993).

CD4 is an accessory molecule required for T-cell activation but it also serves as a receptor for the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), by interacting with the viral surface glycoprotein gp-120 to facilitate its entry into cells (Capon and Ward, 1991). Within the villi, placental macrophages are the only cell type expressing CD4 as this molecule cannot be detected on the ST, CTs and fetal endothelial cells by immunohistochemical staining, Northern blotting or polymerase chain reaction (Lairmore et al., 1993). The levels of CD4 expression on placental macrophages are low compared to adult peripheral blood or cord blood monocytes and can only be detected by sensitive methods (Kesson et al., 1993). Nonetheless, the presence of CD4 suggests that placental macrophages provide a portal of entry for HIV. Indeed, HIV nucleic acids and surface antigens have been detected in Hofbauer cells in an eight-week old aborted fetus (Lewis et al., 1990). Recently, Kesson et al. (1993) showed that selective isolates of HIV were capable of infecting purified placental macrophage cultures and inducing giant syncytia formation such as those seen in infected $CD4^+$ T-lymphocytes (Lifson et al., 1986). However, viral replication as monitored by extracellular p24 antigen over a period of three weeks was significantly lower than in blood monocytes. In a separate study, T-cells co-cultured with infected placental macrophages produced high viral titers, indicative of productive infection (Mano and Chermann, 1991). Therefore, it is likely that placental macrophages, with their reduced capacity to support viral replication and

infectivity, may serve as a sink to trap HIV and prevent them from disseminating to other cells (Kesson et al., 1993). However upon cellular activation, these latent viruses may re-enter the replication cycle to generate progeny and infect other cell types in the villous stroma (Meltzer et al., 1990; Mano and Chermann, 1991).

In addition to the cellular differentiation, the architecture of the stroma changes accordingly during the course of pregnancy. In the first and second trimester, the stroma consists of a network of cells and fibres with fetal endothelial cells, which appear by the end of the second month (Fox, 1978), embedded in between. The interspaces form a series of stromal channels in which Hofbauer cells are suspended. In the last trimester, these channels and the Hofbauer cells gradually disappear and are progressively replaced either by fibres or by sinusoidal enlargement of fetal capillaries (Kaufmann et al., 1977).

C. Cytokines in Placental Villi

The term cytokine was introduced by Cohen to describe soluble mediators produced by lymphoid and non-lymphoid cells that induce specific effects in their target cells (Cohen, Pick and Oppenheim, 1979). These effects are mediated via the binding of cytokines to their corresponding high affinity membrane receptors, thereby initiating a cascade of intracellular transduction signals that include calcium mobilization and the activation of various phospholipases, kinases and phosphatases. These events finally reach the nucleus where they modify gene transcription. The biological activities mediated by cytokines are diverse ranging from cell activation, proliferation, differentiation, alterations in functional and secretory patterns and all the way to cell death. Often such dichotomy of effects is mediated by the same cytokine, the outcome of which depends on its concentration, the locality of its expression and the nature of target cells. In addition, cytokines seldom act as lone-rangers, but rather interact with one another to amplify or dampen the final responses. Thus a network is established among stimulator and target cells in which cytokines serve as a common language to facilitate intercellular communication.

Placenta growth and development is a highly complex and regulated process, demanding the precise integration and co-ordination of cellular activities of the various placental cell types. Thus cytokines, with their pleiotropic effects, appear well-equipped to provide a dynamic network to meet the changing needs with regard to the different gestational periods. It is therefore not surprising that a local cytokine network exists in uteroplacental tissue (Hunt, 1989; Wegmann, 1990; Mitchell, Trautman and Dudley, 1993).

In this section, I shall focus on the localization of cytokines to both hematopoietic and non-hematopoietic cell types within the placental villi. These cytokines described here include the interleukins, the interferons, epidermal growth factor, transforming growth factor-betas, colony stimulating factors and tumor necrosis factors.

The Interleukins

Interleukin 1 (IL-1). IL-1 is a 17.5 kd polypeptide secreted mainly by mononuclear phagocytes but also by fibroblasts, keratinocytes, epithelial cells of the thymus and vascular endothelial cells (Dinarello, 1988). It exists in two forms, α and β, which are translated from two distinct genes on chromosome 2. There is a third structurally related IL-1 molecule known as IL-1 receptor antagonist (IL-1Ra) which competes with IL-1, preventing it from binding to cell surface receptors and triggering the cellular responses mediated by IL-1 (Dinarello and Thompson, 1991). IL-1Ra has a classical secretory leader sequence, which makes it different from both IL-1α and β, which are both synthesized as 31 kd precursor proteins lacking hydrophobic signal peptides. Thus the majority of IL-1α remains in the cytosol, whereas IL-1β is transported out of the cells and cleaved by serine proteases to yield the 17 kd extracellular forms (Dinarello, 1993). Despite a homology of only 26% in their amino acid sequences, IL-1α and β bind to the same receptors, probably because the exon encoding the minimal recognition site for IL-1 receptors (IL-1Rs) is highly homologous between the 2 forms. Two IL-1Rs have been isolated and cloned; the type I receptor (IL-1RI) has a 213 amino acid cytoplasmic domain and is fully functional for signal transduction whereas the type II receptor (IL-1RII) has a short cytoplasmic tail of 29 amino acids and is unclear as to whether it is capable of transducing a signal (Dinarello, 1993).

Both receptors bind the α and β forms of IL-1, as well as IL-1Ra, although IL-1β appears to bind with much higher affinity and selectivity to the type II receptor.

In the placental villi, IL-1α expression has been localized to placental macrophages and endothelial cells in all three trimesters (Berkowitz et al., 1990). Both ST and CTs in first trimester chorionic villi express IL-1β but not IL-1α (Haynes et al., 1993; Simon et al., 1994). *In vitro*, isolated placental cells with macrophage-like properties secrete biologically active IL-1 in culture without further stimulation (Flynn, Finke and Hilfiker, 1982), although Kauma et al. (1992) reported that IL-1 secretion is not an intrinsic property of placental cells, but is induced by the process of cell isolation and culture due to minimal endotoxin contamination.

The expression of IL-1RI has been localized to the ST by immunohistochemical staining (Simon et al., 1994) and functional studies also indicate that trophoblasts respond to IL-1. IL-1α, at doses 50 U/ml and above enhanced hCG secretion and progesterone production by JAR choriocarcinoma cells (Berkowitz et al., 1988; Silen et al., 1989; Hill, 1992). On the other hand, IL-1β has no effect of JAR cells but stimulates hCG production in cell lines propagated from first trimester trophoblasts without affecting their proliferation and fusion to form syncytium (Yagel et al., 1988). High doses (500 U/ml) of IL-1β, however stimulate JEG-3 proliferation (Berkowitz, 1988; Hill, 1992).

IL-1 is also involved in fibrosis and wound repair. Cultured endothelial cells exposed to IL-1 synthesize prostaglandins and platelet activating factors which are potent vasodilators, and upregulate adhesion molecules for leukocyte extravasation. IL-1 also stimulates fibroblast proliferation and the synthesis of type I, III and IV collagen (Dinarello, 1988).

Interleukin 2 (IL-2). IL-2 is a cytokine of 15-20 kd and was first described as an autocrine growth factor for T-cell proliferation. Measurement of IL-2 production in supernatants is generally used as an assay of T-cell activation. IL-2 mediates its effects by binding to the IL-2 receptor (IL-2R) which consists of two chains designated p55 and p70 on the basis of their molecular weights. The p70 chain binds IL-2 with an equilibrium constant (K_d) at 1 nM, and when complexed with p55, the heterodimer binds IL-2 with a 100-fold increase in affinity.

Expression of IL-2 is not restricted to T-cells, since in the placental villi, IL-2 mRNA transcript and protein expression have been localized to the ST (Soubiran, Zapitelli and Schaffar, 1987; Boehm, 1989). The relative abundance of expression is greatest in the first trimester, lowest in the second trimester and with a slight increase seen at parturition. Unfortunately, a recent study by Haynes et al. (1993) failed to confirm these earlier findings as these investigators cannot detect IL-2 mRNA or protein expression in first trimester chorionic villi. There is also no evidence of expression of the p55 subunit of the IL-2R on either CTs or the ST (Soubiran, 1987; Haynes, 1993). Thus it is speculated that if IL-2 is indeed expressed in the ST, it is most likely involved in paracrine interactions with maternal cells in the circulation or in the decidua. Recently, the p75, but not the p55, IL-2R is found to be present on CD56-positive large granular lymphocytes (LGL) in the decidua, the maternal tissue in closest contact with the fetal placenta (Starkey, 1991). In culture, decidual LGL proliferate in response to relatively low concentrations (100 U/ml) of IL-2. Whether these LGL differentiate in response to IL-2 to become lymphokine-activated killer (LAK) cells which may be cytotoxic to trophoblasts remains to be determined.

Interleukin 4 (IL-4). IL-4 is a Th2 cytokine of 15-19 kd. The gene encoding for IL-4 is clustered with genes encoding other growth factors including IL-3, IL-5, colony stimulating factor-1 (CSF-1), granulocyte-macrophage CSF (GM-CSF) and c-fms, the receptor for CSF-1. Its activities include stimulation of fibroblast, B- and T-lymphocyte proliferation and the induction of antibody class switch to secrete IgE and IgG_1 isotypes. The receptor for IL-4 is a 140 kd glycoprotein and is a member of the hematopoietin receptor superfamily whose other members include the p75 subunit of IL-2R, receptors of IL-6, erythropoietin and prolactin. Members of this family are characterized by four conserved cysteine residues in their extracellular domain and a double tryptophan-serine motif (WSXWS) located near the transmembrane region (Idzerda et al., 1990).

IL-4 is not an exclusive T-cell product. In first trimester chorionic villi, IL-4 expression is seen in CTs and the ST (Haynes et al., 1993). The function of this IL-4 is unknown, although IL-4R positive LGL are present in human decidua and IL-4 inhibits their proliferation in the presence of IL-2 (Starkey, 1991); thus, this paracrine interaction suggests a role for IL-4 in regulating the degree of decidual cell activation.

Interleukin 6 (IL-6). IL-6 is a 21-28 kd polypeptide originally identified as a T-cell factor to promote B-cell maturation into antibody producing cells (Kishimoto and Hirano, 1988). Besides Th2 cells, other sources of IL-6 include hepatocytes, mitogen activated B cells, macrophages, fibroblasts and endothelial cells. With such diverse cellular sources, IL-6 is involved in regulating hematopoiesis, coordinating immune and neural functions and mediating acute phase responses during inflammation (Wolvekamp and

Marquet, 1990). The IL-6 receptor (IL-6R) is an 80 kd protein of the immunoglobulin superfamily and it associates non-covalently with a 130 kd glycoprotein which does not bind IL-6 but is required for the biological effects of IL-6.

In human placental villi, IL-6 is localized to the ST in all three trimesters of pregnancy and CTs also stain weakly for IL-6 (Kameda et al., 1990). In addition to secreting IL-6, trophoblasts express IL-6Rs (Nishino et al., 1990). This autocrine interaction serves to increase the production of hCG by cultured trophoblasts when stimulated with IL-1 and TNF-α (Masuhiro et al., 1991; Li et al., 1992). Villous stromal cells, in particular fibroblasts, also produce IL-6 although in significantly less amounts than trophoblasts (Kauma et al., 1993). Nonetheless, IL-6 production by fibroblasts can be enhanced by IL-1β (Kauma, Turner and Harty, 1994).

Interleukin 10 (IL-10). IL-10 was originally isolated and characterized as a factor from murine $CD4^+$ Th2 cells which inhibits cytokine production, particularly interferon-gamma (IFN-γ), by Th1, $CD8^+$ and NK cells (Mosmann and Moore, 1991). Both human and murine IL-10 display extensive homology to an uncharacterized open reading frame in the Epstein Barr virus (EBV) genome (Moore et al., 1990; Vieira et al., 1991). Like murine IL-10, human IL-10 is produced by LPS-activated monocytes, EBV-transformed B-cell lines and Th2 clones, although anti-CD3 activated peripheral blood $CD4^+$ and $CD8^+$ cells, as well as Th0 and Th1-like clones also produce IL-10 (Yssel et al., 1992). Human IL-10 inhibits T-cell proliferation stimulated by either mitogen or anti-CD3 in the presence of autologous monocytes (Taga and Tosato, 1992). The presence of IL-10 also affects a number of macrophage functions. These include downregulation of nitric oxide synthesis leading to impaired

macrophage cytotoxicity against parasites (Gazzinelli et al., 1992), inhibition of the synthesis of IL-1, IL-6, IL-8, IL-10, IL-12 and TNF-α (Mosmann, in press) and downregulation of class II HLA expression on LPS-activated monocytes (de Waal Malefyt et al., 1991). In addition, IL-10 suppression of IFN-γ, IL-2 and lymphotoxin production by Th1 cells is achieved through interfering with the antigen presentation function of macrophages but not B cells (Fiorentino et al., 1991). This suggests that IL-10 could be explored as a potent anti-inflammatory reagent.

In reproductive tissues, IL-10 is localized to the decidual cells of day 6 mouse placentas (Hui et al., 1993). It has been postulated that IL-10 present at the maternal-fetal interface shifts the local immune environment to one biased towards an antibody or Th2 type response, thus dampening the effects of Th1 cytokines such as IL-2 and IFN-γ which are associated with fetal demise (Wegmann et al., 1993). Where IL-10 is localized in human reproductive tissues awaits to be determined; however preliminary *in situ* hybridization studies place some expression at the ST (B. Smith, private communication).

The Interferons (IFNs)

IFNs are generally known for their anti-viral activities although increasing evidence indicates that they also participate in regulating cell growth and differentiation and in modulating the outcome of immune responses. IFNs are divided into two main types: type I interferon (IFN-α and β) is produced by mononuclear phagocytes and fibroblasts whereas type II IFN (IFN-γ) is a product of Th1 cells, natural killer (NK) cells and cytotoxic T-lymphocytes which suppresses humoral immunity by inhibiting the proliferation and cytokine production of Th2 cells (Mosmann and Moore, 1991). Human IFN-α is a multigene family of at least 23 highly homologous

polypeptides, and these species share about 25% amino acid identity with a single human IFN-β subtype. The genes encoding for all IFN-α subtypes and IFN-β are localized on chromosome 9 whereas a separate gene on chromosome 12 encodes IFN-γ. IFN-γ shares little if any sequence homology with IFN-α or -β.

IFN-α and β share a common receptor encoded by a gene on chromosome 6 which translates into a disulfide-linked dimer composed of 51 kd subunits and which associates with a cytoplasmic tyrosine kinase (Langer and Pestka, 1988; Novick, Cohen & Rubinstein, 1994). A separate gene located on chromosome 6 encodes the receptor for IFN-γ, which is a 90 kd transmembrane glycoprotein with equally large extracellular and cytoplasmic domains. The cytoplasmic domain plays an obligate role in receptor-mediated ligand internalization and signal transduction (Farrar, Campell and Schreiber, 1992) whereas the extracellular domain interacts non-covalently with accessory factors encoded by human chromosome 21 in a species-specific manner to yield biologically functional receptors (Hemmi, Merlin and Aguet, 1992). In most cells, the expression of IFN-γ receptors (IFN-γ-R) is constitutive and heir numbers remain unaffected by cellular differentiation or activation (Fulop et al., 1992).

In the placental villi, an antiserum which recognizes at least 8 IFN-α subtypes stains the ST in all trimesters of pregnancy, with reactivity being more prominent in first trimester tissue (Howatson et al., 1988; Bulmer et al., 1990). CTs fail to stain positive for IFN-αs. Hofbauer cells are also a source of IFN-αs, although their reactivity with anti-IFN-α is consistently weaker than that observed in the ST (Bulmer et al., 1990).

Compared to IFN-α, IFN-β staining in the ST is patchy during all three trimesters of pregnancy. Although first trimester villous CTs do not stain for IFN-β, a minority of CTs in the term placenta demonstrate positive cytoplasmic staining (Bulmer et al., 1990). Hofbauer cells do not react with anti-IFN-β.

Both the mRNA and protein for IFN-γ are localized to CTs and the ST in first trimester chorionic villi, with more intense staining in the former tissue (Haynes et al., 1993). Hofbauer cells are also positive for IFN-γ throughout all stages of pregnancy (Berkowitz et al., 1990; Bulmer et al., 1990).

Upon viral stimulation, cultured trophoblasts secrete IFN, with the majority (65%-75%) being IFN-β and the remainder being IFN-α. First trimester trophoblasts secrete higher amounts than term trophoblasts, and the ST also has a higher output than that from CTs (Aboagye-Mathiesen et al., 1993). Since these trophoblast-derived IFNs can protect other cells from viral infection, they may play a role in anti-viral defense when the fetal immune system is not functionally developed.

The placenta is a rich source of IFN receptors. Receptors for IFN-α and -β are present at a concentration of 2.5 fmol/mg tissue (Branca, 1986) and IFN-γ-R are present on the ST and CTs but not on cells in the villous stroma (Valente et al., 1992; Peyman and Haymond, 1992; Hampson, McLauglin and Johnson, 1993). It was reported that a placenta weighing 400 grams contains as many IFN-γ-R as that found on 10^{12} U937 cells (Loke and King, 1990).

IFN-γ is a powerful inducer of HLA class I (the polymorphic HLA-A, -B and -C and the nonpolymorphic HLA-E, -F and -G) and class II antigens (HLA-DP, DQ and DR). IFN-γ upregulates the expression of class I molecules in Bewo and JEG-3 choriocarcinoma cells which generally express low levels

of these antigens. On the other hand, JAR cells, which are devoid of class I antigens, are refractory to the inductive influence of IFN-γ (Anderson and Berkowitz, 1985; Hunt, Andrews and Wood, 1987). Thus it appears that IFN-γ only affects class I expression in choriocarcinomas which are capable of expressing these antigens in the first place (Loke and King, 1990). Accordingly, the ST, which synthesizes little class I HLA mRNA and fails to express detectable cell surface class I HLA antigens throughout all stages of pregnancy (Hunt et al., 1988; Hunt et al., 1990), is unresponsive to IFN-γ despite abundant expression of IFN-γ-R on the ST (Peyman and Hammond, 1992). On the other hand, first trimester villous CTs, which do not stain for cell surface class I HLA molecules, nevertheless transcribe the corresponding mRNA (Hunt et al., 1990). Upon examining the effects of IFN-γ on class I expression in CTs, Hunt, Andrews and Wood (1987) reported that chorionic villous explants from first trimester and term placentas did not respond to IFN-γ by upregulating their class I expression. In contrast, Feinman, Kliman and Main (1987) reported that the addition of IFN-γ increased the staining for class I antigens on cultured trophoblasts isolated from term placenta. These conflicting results could be attributed to the difference in the culture system used. In the tissue explant studies of Hunt, Andrews and Wood (1987), the presence of placental cells other than trophoblasts may downregulate HLA expression or counteract the effects of IFN-γ, a situation that may not be true for CTs isolated from placental villi and placed in culture.

On the other hand, IFN-γ is unable to induce class II HLA-DR expression on trophoblasts both at mRNA (Peyman and Hammond, 1992) and protein level (Peyman, Nelson and Hammond, 1992) because genes encoding these molecules are silenced in both CTs and the ST. It also remains to be

tested whether IFN-α and -β have the ability to upregulate class I expression in human trophoblast cells as both types of IFN have been reported to enhance class I antigens on murine trophoblasts (Zuckerman and Head, 1986).

IFN-γ at high concentration (1000 U/ml) also inhibits the proliferation of JAR, JEG-3 and Bewo choriocarcinomas (Fulop et al., 1992). In the presence of actinomycin D, which blocks RNA synthesis, IFN-γ lyses the cells in a dose-dependent fashion (Fulop et al., 1992). At low concentrations, IFN-γ enhances the metabolic activity of first trimester trophoblasts as measured by the incorporation of ^{3}H-uridine but has no effect on term trophoblasts (Hunt, Andrews and Wood, 1987). Injection of IFN-γ into pregnant mice enhances the rates of fetal resorptions (Chaouat et al., 1990). Targeted disruption of the genes for IFN-γ or IFN-γ-R has no profound adverse or beneficial effects on the fertility of the mice (Dalton et al., 1993; Huang et al., 1993).

IFN-γ also modulates the levels of other cytokine receptors. It downregulates the number as well as the affinity of receptors for epidermal growth factor in human amniotic cells (Karasaki et al., 1989). On the contrary, TNF receptors on various carcinomas are upregulated by both type I and II IFNs (Aggarwal, Eessalu and Hass, 1985; Ruggiero et al., 1986; Billard, Sigaux and Wietzerbin, 1990).

Epidermal Growth Factor (EGF)

EGF is a small 6 kd protein that is a potent stimulator of a variety of normal and neoplastic cells of ectodermal, mesodermal and endodermal origin (Carpenter and Cohen, 1979). In human, the factor was first described as β-urogastrone, an antisecretory protein isolated from urine (Gregory, 1975). This factor demonstrates biological activities similar to those of murine EGF and it also competes with murine EGF in binding to the same receptor (Hollenberg

and Gregory, 1977). Based on these observations, β-urogastrone is recognized as the human counterpart of mouse EGF. The receptor for EGF is a 170 kd glycoprotein which also binds transforming growth factor-alpha (TGF-α) with equal affinity. The cytoplasmic domain of the EGF receptor (EGF-R), which undergoes intrinsic tyrosine kinase phosphorylation upon ligand binding, is highly homologous to the *v-erb-B* oncogene of the avian erythroblastosis virus (Downward et al., 1984).

In the placenta, EGF was reported to be found in the ST, CTs and villous stromal cells by immunohistochemical staining. The intensity of staining in each of these cell types varied with gestation: stronger staining in the trophoblasts than in stromal cells at early gestation and a reversal of the pattern at term (Hofmann et al., 1991). However, using more sensitive techniques such as radioimmunoassay (RIA) and polymerase chain reaction (PCR), Bass et al. (in press) have failed to detect EGF synthesis in either trophoblasts or fibroblasts from first trimester and term placentas. Furthermore, they concluded that the observed positive staining was due to non-specific binding of the polyclonal rabbit anti-EGF antibodies to Fc receptors, as F(ab)' fragments of the antibody failed to stain placental tissue sections or cells but did react with a well-defined positive control.

In the placental villi, EGF-R appears at 6 weeks of gestation (Carson et al., 1983) and also shows a temporal pattern of expression, although with conflicting observations depending on the methods employed for its detection. Autoradiographic studies indicate that [^{125}I] EGF is localized to the ST with very few grains associated with the CT and fibroblasts. In addition, maximal expression of EGF-R occurs at mid-pregnancy (Chegini and Rao, 1985). On the other hand, histochemical studies indicate that first trimester ST stains strongly with anti-EGF-R antibody, which gets weaker in second and term

placentas (Maruo et al., 1987). In contrast, binding studies show that term trophoblasts express the highest number of EGF-R with no significant changes in their binding affinities (Carson et al., 1983; Chen et al., 1988). Furthermore, Chen et al. (1988) showed that isolated trophoblasts, which constituted mostly CTs because the ST was broken up during the isolation process, were capable of binding EGF.

EGF enhances the production of hCG and hPL from both first trimester and term trophoblasts in culture, although with different secretion kinetics (Maruo et al., 1987; Morrish et al., 1987; Barnea et al., 1990). Isolated first trimester trophoblasts in culture do not differentiate into ST (Barnea et al., 1990) whereas term CTs undergo fusion to form syncytium in response to EGF (Morrish et al., 1987).

Because EGF is able to upregulate hCG and hPL production, it is implicated in playing a role in promoting fetal growth and development. It has been reported that in normal pregnancy, maternal levels of urinary and plasma EGF increase, reaching their highest levels in mid-pregnancy whereas mothers carrying intrauterine growth retarded (IUGR) babies achieve only half of those values than women delivering normal infants (Hofmann et al., 1988).

Transforming growth factor-β (TGF-β)

The TGF-β family of proteins consists of closely related members termed β1 to β5 and distantly related members such as Mullerian inhibiting substance and the subunits of inhibins and activins. The characteristic feature of this family is the cleavage of a precursor protein into biologically active forms and the conservation of cysteines in the active peptides (Miller et al., 1990). TGF-βs are secreted by macrophages, LAK cells, T- and B-cells. Although TGF-β1 was first described to transform normal rat fibroblasts to a

neoplastic phenotype, it is now known that it has potent immunosuppressive abilities. It inhibits the production of TNF-α and IFN-γ by human peripheral blood mononuclear cells (Espevik et al., 1987), the generation of cytotoxic T-cells (Ranges et al., 1987), the cytolytic activity of NK cells and diminishes their response to IFN-α (Rook et al., 1986) and suppresses both immediate and delayed type hypersensitivity (Meade et al., 1992). In addition, TGF-βs play a role in the reparative phase of inflammation by promoting angiogenesis and tissue remodeling via stimulating the synthesis of collagen and fibronectin by fibroblasts (Wahl, 1990).

In the developing mouse embryo, the temporal and spatial expression of TGF-β1 appears to correlate with morphogenetic events involving cells and tissues of mesodermal origin, such as the formation of bone and cartilage and the development of cardiovascular structures (Heine et al., 1987). TGF-β1, β2, and β3 have all been detected in mouse placentas by Northern analysis (Miller et al., 1990). In particular, a TGF-β2 like molecule from decidual cells of mid-trimester murine placentas suppress the generation of LAK cells which may be cytotoxic to trophoblasts (Lea et al., 1992).

In first and third trimester human placentas, TGF-βs have been localized to the ST. Villous CTs show weak or absence of staining and no reactivity is observed in the mesenchymal stroma (Vuckovic, Genbacev and Kumar,1992). In terms of trophoblast differentiation, TGF-β1 has been shown to inhibit syncytium formation *in vitro* (Morrish, Bhardwaj and Paras, 1991).

Colony stimulating factors (CSF)

The colony stimulating factors are a group of cytokines named for their ability to stimulate the formations of myeloid colonies, in particular, those containing granulocytes and monocytes, from bone marrow cells (Clark and

Kamen, 1987; Metcalf, 1991). These factors include relatively lineage specific factors such as granulocyte-CSF (G-CSF) and macrophage-CSF, also known as CSF-1 as well as factors that have broader biological activities such as interleukin-3 and granulocyte-macrophage CSF (GM-CSF), encompassing the stimulation of early myeloid progenitors towards the development into neutrophils, eosinophils, macrophages, basophils and platelets. Since this section deals with cytokine expression in the placental villi, I shall confine my discussion to only CSF-1 and GM-CSF as these two are more frequently studied in placental development.

Colony stimulating factor-1 (CSF-1). CSF-1 is a disulfide-linked homodimeric glycoprotein of 45-90 kd which is required for the survival, growth and differentiation of mononuclear phagocytes (Stanley et al., 1983). Cellular sources of CSF-1 include monocytes, fibroblasts and endothelial cells (Clark and Kamen, 1987; Sherr, 1990). Homozygous osteopetrotic (*op/op*) mice, which fail to synthesize biologically active CSF-1 due to a null mutation in the gene (Yoshida et al., 1990; Pollard et al., 1991) are deficient in the number of macrophages in the periphery as compared to their normal littermates (Wiktor-Jedrzejczak et al., 1990). The CSF-1 receptor (CSF-1R) is a 165 kd transmembrane glycoprotein encoded by the *c-fms* proto-oncogene which has intrinsic tyrosine kinase activity. Both CSF-1 and CSF-1R map to chromosome 5 and the genes for CSF-1R and the B isoform of platelet derived growth factor receptor (PDGF-B-R) are arranged in a head to tail tandem array and display strong sequence homology. Given that PDGF-B-R also encodes an intrinsic tyrosine kinase domain, both genes probably arise from duplication of a single ancestral gene (Sherr, 1990).

The importance of CSF-1 in regulating placental growth and development is first established in murine pregnancy. CSF-1 is not expressed in the uteri of virgin mice, but during gestation, the luminal and glandular uterine epithelia synthesize CSF-1 under the hormonal stimulation of progesterone and estradiol (Pollard et al., 1987; Arceci et al., 1989) and the concentration of CSF-1 increases by a thousand fold at the time of parturition (Bartocci, Pollard and Stanley, 1986). Northern blot analysis reveals the preferential expression of a 2.3 kb species of CSF-1 mRNA in the pregnant mouse uteri, in contrast to other CSF-1 producing cells, such as fibroblasts, monocytes or endothelial cells in which the major CSF-1 mRNA species is 4.6 kb. The smaller CSF-1 mRNA lacks the consensus AUUUA sequence that normally confers a short-life on most cytokine messages (Awater, Wisdom and Verma, 1990). This observation suggests that the CSF-1 mRNA synthesized during pregnancy is relatively long-lived and thus may contribute to the sustained CSF-1 production seen during pregnancy (Pollard et al., 1987). Finally, *op/op* mice which are deficient in CSF-1 fail to reproduce when crossed with each other but yield progeny when they are mated with heterozygous partners, indicating a requirement for CSF-1 in pregnancy (Pollard et al., 1991).

During the course of human pregnancy, there is a change in the cell types within the placental villi that express CSF-1 as demonstrated by immunohistochemical staining. In first trimester placenta, CSF-1 is localized mostly to CTs but this expression is no longer detectable by third trimester. Beginning in late first trimester, cells of the villous core, which comprise mostly macrophages and fibroblasts, stain positively for CSF-1. This mesenchymal expression is also transient as by third trimester, the stroma becomes negative for CSF-1. In its place, cells lining the fetal capillaries,

which can be endothelial cells or macrophages, become positive for CSF-1 (Daiter et al., 1992). However, using *in situ* hybridization, Kanzaki et al. (1992) were unable to show the presence of CSF-1 in endothelial cells from third trimester placental samples. Instead, autoradiographic grains of CSF-1 accumulated in stromal macrophages and fibroblasts. In addition, Northern blot analysis also provides supplementary evidence indicating that the main source of CSF-1 from first and term placentas comes from stromal fibroblasts and not from trophoblasts (Harty and Kauma, 1992), and that IL-1β stimulates the production of CSF-1 in fibroblasts (Harty and Kauma, 1992; Kauma, 1993).

In humans, the CSF-1 concentration during pregnancy does not alter as dramatically as it occurs in mice. At term, the concentrations of CSF-1 present in amniotic fluid and serum are only two and four fold higher respectively as compared to those in earlier trimesters. In contrast, first trimester placental tissues contain a two fold higher CSF-1 concentration than those detected in the second and third trimesters (Ringler et al., 1989; Daiter et al., 1992).

The human CSF-1 gene consists of 10 exons that span 21 kb of DNA (Pampfer et al., 1991). Differential splicing of the primary CSF-1 mRNA generates multiple transcripts which upon translation into proteins may differ in their physical and functional properties. For example, the alternative use of various splice acceptor sites in exon 6 can lead to a secreted form of CSF-1 when the intermediate or longest version of exon 6 is used, or a membrane-bound CSF-1 when the shortest version of exon 6 is employed (Rettenmier and Roussel, 1988). The differential usage of the 3'-untranslated exon 9 or 10 generates respectively, a mRNA lacking an AU rich motif or one containing an AU rich sequence at the 3'-terminus (Ladner et al., 1987). In the placenta, the predominant species is 4 kb which contains exon 10 and the long versions

of exon 6 (Daiter et al., 1992; Kanzaki et al., 1992) although other transcripts of smaller sizes are detected at lower levels. Analysis using polymerase chain reaction (PCR) reveals species containing the short exon 6 and exon 9 although the identification of cells expressing these specific mRNAs will require *in situ* hybridization studies with exon-specific probes.

The CSF-1R contains 22 exons and spans greater than 30 kb of genomic DNA (Heisterkamp, Groffen and Stephenson, 1983). In human placental trophoblasts, and the choriocarcinoma cell lines Bewo and JEG-3, transcription of the primary CSF-1R mRNA originates from exon 1 at its 5'-terminus, whereas in monocytes and endometrial cells, a downstream exon 2 which contains the signal peptide sequence is utilized (Visvader and Verma, 1989; Pampfer, 1992). These two are separated by a 26-kb intron and their exclusive expression in either placenta or monocytes is regulated by upstream sequences which are tissue specific (Roberts et al., 1992). Because exon 1 is not translated, the CSF-1R expressed by trophoblasts and monocytes are indistinguishable at the protein level (Rettenmier et al., 1986). In addition, because of the close proximity of the CSF-1R exon 1 and the last exon of the PDGF-B-R, it is suggested that promoter elements required for transcriptional initiation of the placental CSF-1R may lie within the PDGF-B-R gene, thus excluding the co-expression of these two genes in trophoblasts (Pantazis et al., 1991). Indeed it has been observed that PDGF-B-R is preferentially expressed in CTs and CSF-1R in ST (Ohlsson, 1989; Pampfer et al., 1992) and this may be important in regulating trophoblastic differentiation.

The localization of CSF-1R within the placental villi yields controversial results when different sets of antibodies are used in immunohistochemical staining. Pampfer et al. (1992) found strong uniform staining of CSF-1R in ST which persisted until term. Villous CTs also stained

positively but demonstrated a patchy pattern, indicating that not all cells express CSF-1R. In contrast, Jokhi et al. (1993) were unable to localize CSF-1R to the CTs at all trimesters of pregnancy, and they observed that staining in the ST was patchy rather than uniform and the intensity of the staining weakened as pregnancy advanced towards term. The only consistent finding between these two studies was that CSF-1R was expressed by extravillous trophoblasts. The latter study also examined CSF-1R in the stroma and found that CSF-1R staining in Hofbauer cells increased considerably towards term. Since Hofbauer cells also express CSF-1 (Kanzaki et al., 1992), this autocrine interaction may promote their functional maturation as it has been demonstrated that third trimester Hofbauer cells are more competent in antigen processing and presentation (Goldstein et al., 1988).

The overall effects of CSF-1 on the various placental cells are considered beneficial to pregnancy outcome. In the mouse, CSF-1 has been implicated in regulating the proliferation of placental cells (Athanassakis et al., 1987; Wegmann et al., 1989). Macrophages are an important source of cytokines during pregnancy (Hunt et al., 1989), and the maintenance of these cells in the pregnant uteri during gestation appears to involve the presence of CSF-1. Thus, although macrophages of an abnormal phenotype migrate into the uterus of pregnant *op/op* mice on day 7, they disappear by mid-gestation whereas in normal pregnancy macrophages persist in abundance until parturition (Pollard et al., 1991). In human, choriocarcinomas respond to CSF-1 by increased proliferation, and CSF-1 enhance the synthesis of hCG and hPL (Saito et al., 1991; Garcia-Lloret et al., in press) and promote CTs differentiation into ST (Garcia-Lloret et al., in press).

Granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF is a 22 kd glycoprotein produced by activated T-cells, endothelial cells, macrophages and fibroblasts (Clark and Kamen, 1987). Analogous to the IL-2R, the GM-CSF receptor (GM-CSF-R) consists of two subunits, α and β (Hayashida et al., 1990). Transfection studies indicate that the α subunit is an 80-kd glycoprotein which binds the cytokine at low affinity with K_d of 4-6 nM (Gearing et al., 1989; Hayashida et al., 1990; Metcalf et al., 1990). The β subunit by itself does not bind GM-CSF, however upon association with the α subunit, converts it into a high affinity receptor with K_d of 100 pM (Hayashida et al., 1990), although receptors of K_d as low as 40 pM have been detected on myeloid leukemic cell lines (DiPersio et al., 1988). The β subunit is also shared with receptors for IL-3 and IL-5, which possess distinct α subunits that bind the corresponding cytokine (Tavernier et al., 1991; Kitamura, 1991).

GM-CSF was found to be present in human placental conditioned medium (Burgess, Wilson and Metcalf, 1977). Hofbauer and endothelial cells are the major cellular sources independent of gestational age (Berkowitz et al., 1988). The GM-CSF-R is expressed on extravillous trophoblasts and also on membrane vesicles derived from the ST (Loke et al., 1992; Hampson, McLaughlin and Johnson, 1993). On human trophoblasts, a single class of low affinity GM-CSF-R with K_d of 3-5 nM is present (Gearing et al., 1989; Hampson, McLauglin and Johnson, 1993). These low affinity receptors, upon transfection into murine hematopoietic cell lines, are able to deliver a proliferative signal under relatively high concentrations of GM-CSF (Metcalf et al., 1990).

Similar to CSF-1, GM-CSF is viewed as a beneficial cytokine for placental and fetal development (Wegmann et al., 1989; Wegmann, 1990), although knockout mice carrying a null allele for GM-CSF are fertile (Dranoff

et al., 1994). GM-CSF promotes the growth of murine trophoblasts (Athanassakis et al., 1987; Armstrong and Chaouat, 1989) and reverts fetal resorptions to normal levels when injected into pregnant mice known to abort at a high frequency (Chaouat et al., 1990). In murine pregnancy, the main source of GM-CSF is the uterine epithelium (Robertson, Mayrhofer and Seamark, 1992) although GM-CSF transcripts are also localized to maternal decidual cells as well as fetal spongiotrophoblasts (Kanzaki et al., 1991).

In human, GM-CSF stimulates the proliferation of extravillous trophoblasts (Loke et al., 1992). It also enhances production of hCG and hPL and induces the *in vitro* differentiation of semi-purified term CTs into ST (Garcia-Lloret et al., in press).

Tumor necrosis factor

Tumor necrosis factor-alpha (TNF-α). TNF-α is a cytokine of diverse biological activities. It was first identified as a factor in the serum of endotoxin-treated mice that caused the regression of certain transplantable tumors (Old, 1985). Subsequent studies show that TNF-α has multiple effects that encompass cell proliferation, cell death, and the activation and inhibition of cellular functions. Hence, the net biological effects of TNF-α can be either beneficial or detrimental for the host depending on the relative concentration of TNF-α, the duration of cell exposure and the presence of other biological mediators that synergize or antogonize TNF-α activity (Camussi et al., 1990). For instance, the ability of TNF-α to stimulate the production of leukotrienes and platelet activating factors, to increase the adhesiveness of endothelium and to promote leukocyte extravasation constitute part of the host defense mechanisms against infections by bacteria, virus and intracellular parasites, but if the production and activity of TNF-α persist and escape regulation, then

it will contribute to pathological processes as in endotoxic/ septic shock and inflammatory reactions (Beutler and Cerami, 1989).

The active form of secreted human TNF-α is a trimer which consists of three 17 kd subunits arranged in the shape of a triangular pyramid (Hakoshima and Tomita, 1988). The gene for TNF-α resides on chromosome 6, within the HLA-encoding loci (Spies et al., 1986). About 1100 basepairs telomeric of the TNF-α gene is that for lymphotoxin-α (LT-α), also known as TNF-β, a cytokine with a similar spectrum of activities as TNF-α and the two share about 50% homology in their nucleotide sequences. The secreted forms of both cytokines are homotrimers and they do not discriminate in their binding to the two TNF receptors (TNF-Rs), namely p55 and p75. Recent evidence from the X-ray crystal structure of TNF-β bound to the p55 TNF-R show 3 receptor molecules, each binding along the surface groove between two adjacent subunits of the ligand trimer (Banner et al., 1993). Two loop regions identified a-a" and d-e contain important receptor contact sites. By site directed mutagenesis of different positions in the loop within the TNF-α gene, mutants of TNF-α have been generated which bind exclusively to the p55 or p75 TNF-Rs (Loetscher et al., 1993).

In its membrane-bound form, TNF-α exists as a 26 kd type II transmembrane protein and is the precursor of secreted TNF-α (Kriegler, 1988). In contrast, the majority of cell surface LT is a heteromeric complex composed of a monomeric LT-α associated with a dimer of LT-β, a 33-kd transmembrane protein which anchors the entire LT complex to the cell surface (Androlewicz, Browning and Ware, 1992; Crowe et al., 1994). LT-β shares significant homology with TNF-α and LT-α and its gene maps upstream of the TNF-α gene (Browning et al., 1993). Membrane-bound LT

(LT-$\alpha_1\beta_2$) has its own specific receptor possessing a similar structural motif as the two TNF receptors (Crowe et al., 1994). Another difference between TNF-α and LT is their origin: the main source of TNF-α is from activated macrophages whereas lymphotoxin is derived mostly from $CD4^+$ Th1 and $CD8^+$ T-cells (Mosmann and Moore, 1991).

The expression of TNF-α mRNA and protein in cells of the placental villi has been well documented using both *in situ* hybridization and immunohistochemical staining (Chen et al., 1991; Haynes et al., 1993; Yang et al., 1993). In the first trimester, TNF-α mRNA and protein are localized mainly to the ST; staining is low to undetectable in CTs and completely absent in the villous mesenchyme. As pregnancy progresses towards term, the presence of TNF-α in the ST becomes variable whereas mesenchymal cells are stained intensely and consistently by anti-TNF-α antibodies. In addition, higher levels of TNF-α are observed at term than at early stages of pregnancy because Hofbauer cells acquire the capacity to synthesize this cytokine.

The pleiotropic effects of TNF-α can be partly attributed to the presence of TNF-Rs on nearly all cell types with the exception of erythrocytes and unstimulated T-lymphocytes (Fiers, 1991). The presence of TNF-Rs is a requisite for a biological effect (Engelmann et al., 1990). Two high affinity TNF-Rs have recently been isolated and cloned (Loetscher et al., 1990; Smith et al., 1990) and specific antibodies have been generated to recognize them (Brockhaus et al., 1990; Thoma et al., 1990). TNF-RI has a molecular weight of 55 kd, thus is known as p55 or p60, and its K_d for TNF-α is about 0.5 nM. TNF-RII has a molecular weight of 75 kd, thus is referred to as p75 or p80, and its K_d for TNF-α is about 0.1 nM. The two receptors share 28% homology in their extracellular domain which is composed of four cysteine-rich repeats, each being 40 residues in length. The location of these cysteines is extremely

well conserved, suggesting that conserved disulfide bonds are critical for the maintenance of this structural motif (Tartaglia and Goeddel, 1992). Similar homologous repeats are found in the extracellular domains of the low affinity receptors for nerve growth factor, the newly discovered receptor for cell-surface LT-β, the putative B-cell cytokine receptor CDw40, the rat OX40 T-cell activation antigen and the Fas antigen (Smith, Farrah and Goodwin, 1994; Beutler and Van Huffel, 1994). Soluble forms of both receptors also exist in human cell culture medium (Kohno et al., 1990), serum (Schall et al., 1990) and urine (Engelmann et al., 1989) and are suggested to play a major role in regulating the levels of bioactive TNF. For instance, they could compete for binding with cell surface receptors and thereby antagonize the cellular effects of TNF (Mohler et al., 1993), or they could bind TNF in a dissociable form, thereby affecting the pharmacokinetics and stability of TNF (Fiers, 1990)

Both types of TNF-Rs are expressed in cells of the placental villi although differences related to gestation stages and cell lineages are evident by in situ hybridization and immunohistochemical staining methods (Yelavarthi and Hunt, 1993). In first trimester tissue, the p55 TNF-R mRNA is detected strongly in both macrophages and endothelial cells; the protein, however, is present at low levels on these cells. In contrast, mRNA expression in the villous ST and CT is patchy and the intensity of signals varies even within a single villous; nonetheless, ST stains strongly positive for the protein. In term placentas, the expression of p55 TNF-Rs is still strong in the stromal mesenchyme, and there is greater uniformity of staining in the ST, although it is much weaker compared to the staining in placental macrophages.

In contrast to p55, which appears to be ubiquitously expressed in both mesenchymal and trophoblastic lineages during the course of gestation, the expression of p75 TNF-Rs in placental cells is dependent on the stages of

pregnancy (Yelavarthi and Hunt, 1993). Thus in first trimester, p75 transcripts and the corresponding protein are detected in intermittent stretches of the ST, and low in CTs and mesenchymal cells. At term, a reversal in the pattern of expression is observed: the intensity of signals is strongest in the stroma, particularly in the fetal endothelial cells (Hampson, McLaughlin and Johnson, 1993) whereas the ST has a reduced capacity to synthesize the p75 receptor. In addition to these high affinity receptors, low affinity receptors with K_d of 20 to 30 nM have been reported on the membranes of ST and their numbers appear to exceed those of the high affinity receptors (Eades, Cornelius and Pekala, 1988; Hampson, McLaughlin and Johnson, 1993). Soluble receptors of p55 and p75 are also detected in the urine and serum of pregnant woman, with p55 levels increasing during the course of gestation, then decreasing in association with spontaneous delivery (Austgulen et al., 1992; Austgulen et al., 1993).

The effects of TNF-α on placental growth and fetal development have largely been negative. Studies in rodents indicate that the levels of TNF-α mRNA expression in placental tissues derived from pregnant mice with high rates of spontaneous fetal resorptions are greatly elevated as compared to those from normal mating combinations (Tangri and Ragupathy, 1993). Injection of TNF-α into pregnant rats (Silen et al., 1989) as well as into pregnant mice (Chaouat et al., 1990) results in placental necrosis and fetal demise. In addition, LPS-induced fetal resorption, likely to be mediated by TNF-α, is reversed by pretreatment of the animals with the TNF-α suppressing drug pentoxifylline (Gendron et al., 1990). In humans, placental/decidual production of TNF-α in response to local microbial infections has been implicated in the pathogenesis of preterm labor (Casey et al., 1989; Romero et al., 1989; Hillier

et al., 1993) and elevated TNF-α in mid-trimester amniotic fluid is associated with IUGR infants (Heyborne, Witkin and McGregor, 1992).

The effects of TNF-α on placental cells are mostly inhibitory. High concentrations of TNF-α inhibit the proliferation of the JEG-3 choriocarcinoma cell line (Berkowitz et al., 1988). TNF-α inhibits DNA synthesis in rat trophoblast cell lines (Hunt, Atherton and Pace, 1990) and rat labyrinthine trophoblasts, which are the equivalent of human villous trophoblasts, constitute the primary sites of injury after LPS and TNF-α administration (Silen et al., 1989). However, the presence of TNF-α in utero-placental tissues does not always correlate with deleterious effects since TNF-α is expressed in non-diseased utero-placental tissues (Bulmer et al., 1990; Chen et al., 1991). TNF-α also serves as an autocrine growth factor for the JAR choriocarcinoma cell line (Yang et al., 1993). Mice deficient in the expression of p55 TNF-Rs are normal in terms of fertility (Pfeffer et al., 1993; Rothe et al., 1993).

Table 1.1 summarizes the distribution of cytokines in the various cells of the placental villi. In this discussion, I have omitted the more recently discovered cytokines IL-11 to IL-13 because their effects on human placental cells have not been fully investigated. In addition, other growth factors such as PDGF and insulin growth factors (Hill et al., 1993) have recently been localized to cells of the placental villi, and their effects on placental cells also await to be explored.

Table 1.1. Localization of cytokines and their corresponding receptors to cells of the human placental villi.

Cytokine	Localization	Localization of receptor
IL-1α	Mϕ, EC	CT, ST
IL-1β	ST, CT	FB
IL-2	ST	LGL
IL-4	CT, ST	LGL
IL-6	ST, CT, FB	CT, ST
IL-10	ST	ND
IFN-α	ST, Mϕ	ND
IFN -β	ST, CT	ND
IFN-γ	CT, ST, Mϕ	ST, CT
EGF	Mϕ, EC	ST, CT
TGF-β	ST	ND
CSF-1	CT, Mϕ, EC, FB	ST, Mϕ
GM-CSF	Mϕ, EC	ST
TNF-α	ST, CT, Mϕ	ST, CT, Mϕ, EC

Abbreviations :

CT: Cytotrophoblast; EC: Endothelial cell; FB: Fibroblast; LGL: Large granular lymphocyte; Mϕ: Macrophage (Hofbauer cell); ND: Not determined; ST: Syncytiotrophoblast

CHAPTER II
HYPOTHESIS, RATIONALE AND PROJECT

A. Hypothesis

Lymphohematopoietic cytokines were originally identified for their role in regulating the proliferation, differentiation and function of cells of the lymphoid and myeloid lineages. However, it is now recognized that cytokines are not exclusive products of hematopoietic cells, but rather they behave as autocrine, paracrine and juxtacrine signals to facilitate intercellular communication. Localized cytokine networks can be established among hematopoietic and non-hematopoietic cells within specialized tissues such as the lungs (Kunkel et al., 1991) and the skin (Luger and Schwarz, 1990). As mentioned in the previous section, a cytokine network also exists within the placental villi, and a large body of literature regarding the identification and localization of these cytokines and their corresponding receptors is readily available. However, our knowledge on how cytokines modulate placental growth and development via their effects on the various placental cell types is still limited. ***My general hypothesis is that cytokines in the placental villi act very much as double-edged swords; their effects can be beneficial or harmful depending on the timing and location of their expression. The specific hypothesis is that TNF-α has a specific effect on trophoblasts, that this effect involves cell death and that unregulated expression of TNF-α contributes to pathological manifestations within the placenta.***

B. Rationale

For this thesis project, I decided to study the effects of TNF-α on primary cultures of purified trophoblasts for the following reasons :

1. Trophoblasts constitute the principal cell type at the maternal-fetal interface. Throughout placental development, different types of trophoblasts emerge with differing structural and functional specialization to satisfy the demands of the fetus at various stages of gestation. For instance, extravillous trophoblasts penetrate the maternal endometrium to anchor the embryo in its proper position and subsequently differentiate into interstitial trophoblasts and placenta bed giant cells depending on their depths of penetration. On the other hand, villous trophoblasts carry out metabolic and synthetic functions as well as facilitate gaseous and nutrient exchange between mother and fetus. Despite such variability, all trophoblasts are proposed to trace their origins to a layer of primitive mononucleated CTs, the stem cells which differentiate by as yet undefined mechanisms to give rise to the villous and extravillous lineages (Aplin, 1991).

2. Villous CTs are the progenitor cells which give rise to the ST. The ST normally provides a continuous barrier segregating maternal and fetal tissues, and contributes to the synthesis of steroid and peptide hormones. As the ST is a terminally differentiated cell with limited life-span, its regeneration is dependent on the fusion of the underlying layer of CTs and therefore TNF-α effects on CTs will also affect the integrity of the ST. Since the apical surface of the ST is directly bathed in maternal blood, and is therefore fully accessible to any cell in the maternal circulation including those harboring infectious agents, I propose that depletion of this pool of progenitor CTs will

hamper regeneration of the ST and lead to a defective barrier because of compromised repair. Loss of barrier function would hence facilitate the transplacental transmission of infectious agents.

3. Primary cultures of purified trophoblasts or trophoblastic-like cell lines permit the study of cytokine effects on placental trophoblasts free from contamination by stromal and blood cells. Indeed, choriocarcinomas have often been used as a model for trophoblasts, and they probably provide an adequate model for the study of extravillous trophoblasts because they both proliferate and express HLA-G (Kovats et al., 1990; Hunt and Orr, 1992). However, the results derived from choriocarcinomas may not be fully applicable to villous trophoblasts isolated from term placentas because the latter are deficient in class I HLA expression and proliferative capacity. I therefore decided to initiate a study about cytokine effects on villous trophoblasts using primary cultures of purified CTs. There are relatively few studies addressing the effects of cytokines on trophoblasts using highly purified primary human placental CTs, nor have cultures of highly purified primary CTs been characterized in detail. Furthermore, a purified population of primary CTs can be applied to address questions regarding the ST when improved methods of CT differentiation into ST become available, as intact ST is difficult to obtain because the tissue is broken up during the process of cell isolation.

Among the different types of cytokines, I decided to investigate TNF-α for the following reasons :

1. Trophoblasts express high and low affinity TNF-Rs and therefore have the potential to respond to TNF-α. TNF-α is synthesized during early pregnancy by trophoblasts and in later gestation stages by Hofbauer cells.

Thus paracrine and autocrine interactions may result in differential responses by trophoblasts at early and late stages of pregnancy.

2. At term, the levels of TNF-α are higher as compared to earlier stages of pregnancy. Coincidentally, there is a decrease in the population of CTs as pregnancy advances toward term. Does this temporal expression of TNF-α have any relation with depletion of the CTs given they are in the vicinity of TNF-α producing placental macrophages? If so, what are the effects of TNF-α on these CTs? TNF-α is well-known for its cytotoxicity on tumor cells (Sugarman et al., 1985) and a few primary cells, such as oligodendrocytes (Selmaj et al., 1991) and pancreatic β-cells (Rabinovitch et al., 1990); so there exists the possibility that the disappearance of CTs might occur through the cytotoxic action of TNF-α. If this is true, by what mechanisms is the cytotoxic signal transmitted in CTs?

3. TNF-α has been implicated in contributing to pre-term labor and IUGR (Casey et al., 1989; Romero et al., 1989; Heyborne et al., 1992; Hillier et al., 1993). Are these due to the direct action of TNF-α on trophoblasts or via indirect action on other cells within the placenta?

C. Experimental approach

My approach to study the effects of TNF-α on trophoblasts is to use a simple *in vitro* culture system of purified CTs free from contamination by fibroblasts, macrophages and other stromal or blood cells. Although there are drawbacks in this system, such as the lack of a basal culture surface characteristic of epithelial trophoblasts *in vivo*, this simple model of cultured trophoblasts nonetheless permits a convenient way of addressing my experimental questions. Furthermore, it is possible to improve such a model

by the addition of amnion membranes (Bullen et al., 1990) or culture inserts to induce a polarized/ two-sided surface.

Because of the heterogeneity of placental cells, and the insufficiency of existing purification methods, I first developed a protocol to purify CTs from term placentas and then proceeded to define their properties in culture. The purification scheme and characterization of the purified cells in culture form the core of the study described in Chapter IV.

Having defined an *in vitro* culture system whereby CTs remained mononucleated, I then proceeded to investigate the effects of TNF-α on cultured CTs by the addition of recombinant TNF-α into the system. Because IFN-γ is known to synergize with many of the effects of TNF-α, it is also included in the study (Chapter V).

These experiments led to the discovery that TNF-α mediates apoptosis in trophoblasts which can be further enhanced in the presence of IFN-γ. My third objective was therefore to investigate the mechanism associated with TNF-α-induced trophoblast cell death (Chapter VI) and finally, to search for biological agents that inhibit TNF-α-induced apoptosis of cultured CTs (Chapter VII).

CHAPTER III
MATERIALS AND METHODS

A. Materials and Methods (General)

Isolation of placental cells from human term placentas. Human term placentae from spontaneous term vaginal deliveries or elective cesarean sections at term were used as a source of placental cells. In both cases, the placentae used lacked gross calcification and the cells derived from either preparation showed no distinct differences in terms of viability. Examination for pathological parameters was not performed on a routine basis. Suspensions of placental cells were prepared from the chorionic villi using a modification of a previously described method (Morrish et al., 1987). Briefly, the tissue was subjected to seven trypsin-DNase digestions using 0.25% trypsin from porcine pancreas (ICN Biomedicals, Aurora, Ohio; *Note*: trypsin strength may vary from batch to batch and therefore screening was required to determine the optimal batch) and 1000 units/ ml of DNase I from bovine pancreas (Sigma, St. Louis, MO), and cells from fractions 3 to 7 were pooled. Cells were then washed twice in PBS/ 2% fetal calf serum (FCS) (Hyclone, Logan, Utah), and resuspended in lysis buffer (150 mM ammonium chloride, 10 mM sodium bicarbonate, and 0.1 mM disodium EDTA) with constant inversion for 10 minutes at room temperature (RT) to remove erythrocytes. After washing twice with PBS/ 2% FCS, the cells were ready for the column purification described below. On average, between 100 to 180 million cells were obtained from 30 g of wet tissue.

Purification of placental cells via negative selection. Plastic columns were filled with 1 ml of goat anti-mouse immunoglobulin (Ig)-conjugated glass beads prepared according to the manufacturer's recommended procedure (Biotex Laboratories Inc., Edmonton, Alberta). Meanwhile, placental cells were incubated in PBS/ 20% normal goat serum for 20 minutes on ice to reduce non-specific binding. The cells were then washed, resuspended in PBS/ 2% FCS and incubated with mouse-anti-human CD9 antibody 50H.19 (MacLean et al., 1982; Morrish et al., 1991) at a ratio of 1 μg antibody to 2 million cells. Commercially available anti-CD9 antibody (Binding Site, Birmingham, UK) has also been tested and found to yield similar results to 50H.19. After a 40-minute incubation on ice, the cells were washed twice with cold PBS/ 2% FCS and resuspended to give 10 million cells per ml in PBS/ 2% FCS. This preparation was then loaded onto PBS washed columns and the cells eluted at a flow rate adjusted to 1 drop every 8 seconds. Between 40 to 60 million cells can be loaded onto each column without impeding the flow rate. Directly after loading, non-adhering cells were eluted with 13 ml of PBS/ 2% FCS. These cells were centrifuged, resuspended in FCS at 10 x 10^6 cells/ ml and cryopreserved in 10% (v/v) dimethylsulfoxide in FCS under liquid nitrogen.

Culturing of column-purified cells. Cryopreserved cells were thawed in a 37°C water bath, washed twice by centrifugation with cold Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Grand Island, NY), resuspended in IMDM supplemented with 10% (v/v) FCS, 50 μg/ml gentamycin (Gibco, G.I., NY) and plated 1x10^5 cells per 100 μl per well in Linbro 96-well plates (Flow Labs, Virginia). This concentration was found to give a confluent monolayer when the cells were optimally isolated and therefore was used throughout the

experiments unless specified otherwise. After a 4 hour incubation at 37°C in 5% CO_2 to facilitate adherence, non-adherent cells were removed by 3 washes with warm IMDM. Adherent cells were maintained in IMDM/ 10% FCS and the medium was changed every 48 hours. *In all experiments, cytokines or other reagents were added to the trophoblasts after the 4-hour adherence step and after washing away the non-adherent cells.*

Cytokines added to cultures. Human recombinant TNF-α was a kind gift from Hofmann LaRoche (Basel, Switzerland). IFN-γ was purchased from Collaborative Biomedical Products (Bedford, MA) and used at 100 U/ml according to the supplier's recommendations. Human epidermal growth factor (EGF) was purchased from Pepro Tech Inc. (Rocky Hill, NJ) and used at 10 ng/ml.

MTT Assay. This was based on the method of Mosmann (1983). Supernatants were aspirated and the cultures in 96-well plates were replenished with 90 µl of IMDM/ 2% FCS followed by the addition of a 10 µl aliquot of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) at 5 mg/ml. After a 4 hour incubation period, 150 µl of 0.04 M HCl in isopropanol was added and pipetted vigorously to dissolve the crystals. The photometric measurement was read at 570 nm and a reference wavelength at 650 nm on a UVmax microplate reader (Molecular Devices, Menlo Park, CA).

Assay for DNA content. The assay is a modification of the method described by Cesarone, Bolognesi and Santi (1979). Cells cultured in 96-well plates were washed twice with PBS to remove residual phenol red in the culture medium and 50 µl of 0.1 M NaOH was added to each well to lyse the

cells. 50 µl of HCl-Tris buffer (0.1 M HCl-Tris pH 8.0 and 0.2 M HCl in a 1:1 volume ratio) was added to neutralize the sample. The contents of each well (100 µl) was mixed with 1.9 ml of Hoechst Dye solution (0.1 µg/ml Hoechst 33258 [Sigma Chemical, St. Louis, MO] , 10 mM Tris, 1mM EDTA, 2.1 mM NaCl, pH 7.4) and the fluorescence quantitated on a LS-5 Luminescence Spectrometer (Perkin Elmer, Norwalk, CT) using calf thymus DNA as a standard.

Detection of apoptotic cells using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). Trophoblasts were fixed with acetone : methanol (1:1) for 10 minutes at RT, washed 3 times with PBS, then subjected to TUNEL as described by Gavrieli, Sherman and Ben-Sasson (1992). The fixed cells were incubated in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride) for 10 minutes at RT. A mixture of bio-16-dUTP (Sigma B3029, St. Louis, MO) at 16.5 µM, dATP (Boehringer Mannheim, Laval Quebec) at 16.5 µM and TdT enzyme at 5 U/µl (Boehringer Mannheim) in TdT buffer were added and the reaction was allowed to proceed for 60 minutes at 37°C. The reaction was terminated by adding 2X SSC (300 mM sodium chloride plus 30 mM sodium citrate). After washing the cells 3 times in double distilled water (DDW), endogenous peroxidase activity was quenched in a 3% H_2O_2 solution for 15 minutes at RT. Cells were then rinsed in PBS and blocked with 3% skim milk powder in PBS containing 0.5% Tween 20 for 15 minutes at RT. The cells were incubated for 30 minutes at RT with ExtrAvidin peroxidase (Sigma) diluted 1:50 in blocking buffer, washed 3 times with DDW, then stained with aminoethyl carbizole at RT until a red color developed (approximately 10 minutes). Positive controls were generated by incubating the fixed cells in 1

μg/ml DNase I (Sigma 5025, St. Louis, MO) prior to labeling whereas negative controls involved carrying out the TUNEL procedure without the addition of bio-16-dUTP.

Statistical analysis. The Student's paired *t*-test (Microsoft Excel 5.0) or the Tukey's multiple-comparison test (Byrkit, 1987) was used to determine the statistical significance between compared groups.

B. Materials and Methods Specific for Chapter IV

Preparation of tissue sections from term placenta. Frozen sections (6 μm thick) were cut from term placenta, placed onto Aptex-coated slides and stored at -70°C until needed. Before immunohistochemical staining (see below), they were thawed at RT and fixed for 20 minutes in absolute methanol at -20°C.

Preparation of placental macrophages. Macrophages were isolated from pre-column placental cell suspensions (see Section A) by a brief adherence on tissue culture treated plastic plates. Cells were plated at 2 x 10^6 per ml in IMDM/ 10% FCS and incubated for 30 minutes at 37°C. Plates were then vigorously washed to remove non-adherent and loosely adherent cells and refed with IMDM/ 10% FCS with 10 ng/ml of recombinant human CSF-1. These cell preparations were invariably used within 6 days of isolation to avoid fibroblast overgrowth. Purity was assessed by immunostaining with anti-Leu M5, a marker for mononuclear phagocytes (>85 % cells positive); anti-CD 14 (>79% positive); anti-HLA class I (>99% positive) and anti-HLA class II (>82% positive). Over 80% of the cells isolated in this way were phagocytic and strongly reduced

nitroblue tetrazolium, providing functional evidence of their macrophage phenotype.

Preparation of placental fibroblasts. In order to obtain pure populations of placental fibroblasts, pre-column cell suspensions were plated at 1 x 10^6 cells/ ml in IMDM/ 10% FCS for 2 hours, extensively washed and maintained in the same medium. Under these conditions, fibroblast overgrowth became apparent after 8-10 days of culture and after 2-3 weeks, almost pure populations of morphologically distinct colonies of spindle cells with prominent nucleoli were observed. Phenotypically these cells are non-phagocytic, >99% vimentin positive, >99% HLA class I positive, >99% CD9 positive, <2% Leu M5 positive and <1% cytokeratin positive. Placental fibroblasts can be maintained routinely for at least 10 passages without loss of viability.

Flow cytometic analysis of placental cells. One million cells in a 50 µl volume of PBS were incubated with an equal volume of primary antibody diluted in PBS containing 20% goat serum for 45 minutes on ice. After washing, the cells were resuspended in 50 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Becton-Dickinson, New Jersey, USA) for an additional 45 minutes on ice. The cells were then fixed in 1 ml of 1% formalin in PBS and transferred to 12 x 75 mm Falcon tubes (Becton-Dickinson) for analysis on a FACSCAN machine using the supplier's software program LYSYS II (Becton-Dickinson, San Jose, CA).

The primary antibodies used in this study were as follows: 50H.19 (anti-CD9) diluted 1:100 to give 50 µg/ml of Ig; the monomorphic anti-MHC class I antibody, W6/32, which identifies a shared determinant on HLA-A, B, C and G

was obtained from Dimension Laboratories Inc, (Mississauga, Ontario) and used at a 1:100 dilution (20 μg/ml). Both anti-human Leu M5 (Becton-Dickinson) and culture supernatants of trophoblast specific antibody GB25 (Hsi and Yeh, 1986; kindly provided by Dr. B.L. Hsi, INSERM 210, Nice, France) were used at a 1:6 dilution. Controls Igs consisting of purified mouse myeloma proteins of the isotypes IgG_1 (for GB25) and IgG_{2a} (for 50H.19, W6/32 and Leu M5) were obtained from Zymed (San Francisco, USA) and diluted to give the same concentration as their controlled partners.

Immunohistochemical staining of cultured placental cells. Placental cells cultured in Linbro 96-well plates were washed with PBS and fixed in 100% methanol at 4°C for 10 minutes. The staining was performed according to the directions supplied by the Histostain Kits purchased from Zymed (San Francisco, USA). Briefly, the fixed cultures were washed in PBS for 10 minutes and endogenous peroxidase activity was quenched in 3% hydrogen peroxide in absolute methanol. Non-specific binding was blocked with normal rabbit serum and the cells were incubated for 1 hour at RT with the following primary mouse antibodies: anti-cytokeratin (undiluted; Becton Dickinson); anti-vimentin (1:50; Dakopatts, Glostrup, Denmark) and anti-CD9 (50H.19; 100 μg/ml). Monoclonal anti-desmosomal protein (Clone ZK-31) was purchased from Sigma (St.Louis, MO) and used at a 1:100 dilution.

Rabbit anti-hCG-β chain (Dakopatts, Glostrup, Denmark) was used at a 1:100 dilution. Controls were incubated with the Ig fraction from non-immunized rabbits (Dakopatts).

The reaction was visualized by using streptavidin-biotin peroxidase reagents supplied by the Histostain kit. Positive reactions yielded a red colour

and the cells were subsequently counterstained with hematoxylin. Photographs were taken with a Leitz inverted photomicroscope.

Immunofluorescent staining of cultured placental cells. Post-column cells were cultured overnight in 16 well Lab-Tek glass chamber slides (Nunc, Naperville, IL) coated with 10 μg/ml of bovine type IV collagen (Sigma, St. Louis, MO) to allow attachment of cells onto the glass surface. The cells were fixed in acetone at -20°C for 10 minutes, then washed in PBS for 5 minutes. Cells were incubated with the above mentioned anti-cytokeratin antibody for 30 minutes at 37°C followed by 3 washes in PBS. FITC conjugated goat-anti-mouse Ig, diluted 1:10 in PBS, was added and incubated for a further 30 minutes at 37°C. The slides were washed in PBS, mounted with a 5% solution of n-propyl gallate in glycerol, and photographed with an Olympus IMT inverted microscope equipped with epifluorescence optics.

Measurement of hCG production. Supernatants from column purified placental cells cultured with 25 ng/ml of recombinant human GM-CSF (a gift from Sandoz, Basel, Switzerland) were assayed for hCG using the Clinical Assays Gamma coat™ [^{125}I] hCG Immunoradiometric Assay Kit (Incstar Corporation, Stillwater, MN). Briefly, triplicates of 100 μl aliquots of hCG blank and standards (0, 5, 10, 25, 100, 200, 300 mIU/ml) as well as supernantant samples were added to tubes coated with rabbit anti-hCG. To each of these tubes, 100 μl of hCG IRMA assay buffer was added and the tubes were incubated at 37°C for 45 minutes. After washing the tubes 3 times with 3 mls of buffer, 200 μl of [^{125} I] anti-hCG IRMA tracer was added to all the tubes and incubated for a further 30 minutes at 37°C. After 3 washes, each tube was counted for one minute in a gamma counter (1271 CliniGamma,

LKB-Wallac, Turku, Finland). The concentration of hCG in the supernatant was determined from the standard curve.

Assay for protein content. Cells cultured in 96-well plates were washed in PBS to remove all traces of medium. A 25 µl volume of 0.5% Triton X-100 (Sigma) was added to lyse the cell membranes and the plates were incubated for a further 15 minutes on ice. After careful mixing, a 10 µl aliquot was then removed and 200 µl of BCA protein assay reagent (Pierce, Rockford, IL) was added. The plates were then incubated at 60°C for 30 minutes and allowed to cool to RT before the absorbance measured at 570 nm. Bovine albumin fraction V (Sigma) was used as a standard.

Proliferation assay for trophoblasts. Column-purified trophoblasts were cultured in Linbro 96-well plates as described above. On days 1, 7 and 14 of culture, a 20 µl aliquot of tritiated thymidine (^{3}H-TdR, New England Nuclear, Boston, MA, specific activity = 20 Ci/mmol) was added to the wells to give a final concentration of 1 µCi/well. Cells were then incubated for 4 hours and then 0.05% Triton X-100 was added for 15 minutes at RT to allow strongly adherent cells to be removed from the plates during harvesting. Cells were harvested on glass fibre filters (Packard, Meriden, CT) using a Micromate 196 cell harvester (Canberra Packard). Tritium radioactivity was quantitated on a MATRIX 96 Direct Beta Counter (Canberra Packard, Meriden,CT).

C. Materials and Methods Specific for Chapter V :

Flow cytometric analysis of DNA content using propidium iodide. This was based on the method by Crissman and Steinkamp (1973). Non-adherent

trophoblasts from the supernatants of cultures treated with TNF-α plus IFN-γ were harvested and resuspended in 2 x 10^6 cells/ml of cold PBS, followed by a slow addition of 5 volumes of cold 70% ethanol. The cells were then fixed for 30 minutes on ice. As a standard for normal, non-proliferating cells containing a 2n DNA content, freshly thawed trophoblasts were plated for 4 hours to allow adherence, then washed to remove non-adherent cells. The adherent trophoblasts were then detached using trypsin-EDTA and fixed according to the above procedure. The samples were stained for 20 minutes in PBS containing 100 μg/ml of RNase A (Boehringer Mannheim Canada, Laval, Quebec) and 10 μg/ml of propidium iodide (Sigma, St. Louis, MO) at RT in the dark. The samples were stored overnight and analyzed on a FACSCAN using the LYSYS II program with an excitation wavelength of 488 nm and the voltage for fluorescence set at 440 V.

DNA fragmentation assay. This method was based on the protocol of Swat, Ignatowitz and Kisielow (1991). Cells from control and cytokine-treated culture supernatants were harvested and spun at 400g. The pellet was resuspended in 35 μl of lysis buffer consisting of 20 mM EDTA, 100 mM Tris (pH 8.0), 0.5% sodium lauryl sarcosinate and 7 mg/ml proteinase K (Gibco BRL, Gaithersburg, MD). The samples were heated at 50°C for at least 2 hours, and 10 μl of 1 mg/ml RNase was added and then incubated for a further 2 hours. Electrophoresis was carried out on a 1% agarose gel that was afterwards stained with 0.1% ethidium bromide for 4 hours before visualizing the DNA bands under UV.

Combined TUNEL with desmoplakin staining. Column-purified cells were cultured in Linbro-96 well plates as described in Section A. EGF (10

ng/ml) was added for 7 days (with medium changes every two days) to induce syncytium formation. After one week in culture, cells were then washed extensively, refed with IMDM/ 10% FCS and incubated for 4 hours to remove any residual EGF. TNF-α (50 ng/ml) and IFN-γ (100 U/ml) were then added and incubated for 18 hours. The cultures were washed once, fixed in acetone: methanol (1:1) and processed for TUNEL staining for apoptotic nuclei as described in Section A up to the ExtrAvidin step. After washing the peroxidase away in distilled water, the wells were blocked with goat anti-mouse serum from the Histostain Kit (Zymed, San Francisco, CA), then stained for one hour with anti-desmoplakin antibody (Sigma, St. Louis, MO) at a 1:100 dilution. Subsequent steps were performed according to the directions and reagents supplied by the kit. Photographs were taken with a Leitz inverted photomicroscope.

D. Materials and Methods Specific for Chapter VI:

Anti-TNF receptor (TNF-R) antibodies. Neutralizing monoclonal antibodies against the p55 TNF-R (designated htr-9) and the p75 TNF-R (designated utr-1) were kindly provided by Dr. Loetscher (Hofmann LaRoche, Basel, Switzerland). Both belong to the mouse IgG_1 isotype (Brockhaus et al., 1990).

Receptor-type specific TNF-α mutants. These TNF-α mutants were a kind gift from Dr. Loetscher (Hofmann LaRoche, Basel, Switzerland). They were generated by site directed mutagenesis and expressed as recombinant proteins in *Escherichia coli* as described elsewhere (Loetscher et al., 1993). The p55 specific mutant has a double mutation, one at position 32 replacing

arginine by tryptophan and the other at position 86, replacing serine by threonine and is therefore designated R32W-S86T. These changes result in a loss of binding for p75 while retaining affinity for p55. The p75 specific mutant also has two mutations, one at position 143 from aspartic acid to asparagine and the other at position 145 replacing alanine by arginine, therefore designated D143N-A145R. This mutant shows a 5 to 10 fold lower binding affinity for p75 with respect to wild type TNF-α but fails to bind to p55.

Cell culture in arginine deficient medium. Column purified cells were plated at $1x10^5$ cells per 100 μl of IMDM plus 10% FCS in Linbro 96-well plates and allowed to adhere by a 4 hour incubation at 37°C in 5% CO_2. Non-adherent cells were removed by 3 washes with warm RPMI lacking L-arginine (Select Amine Kit, GIBCO, Grand Island, NY). Increasing concentrations of L-arginine plus 10% dialyzed FCS were added to the above RPMI medium. The cytokines TNF-α and IFN-γ were prepared in RPMI plus 10% dialyzed FCS with different concentrations of L-arginine, added to cultures and incubated for 4 days. At the end of the culture period, viability was determined by the MTT assay as described above.

Inhibitors of nitric oxide (NO) production. N^G-monomethyl-L-arginine, monoacetate salt (NMMA) was purchased from Calbiochem (San Diego, CA) and dissolved in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY) to give a stock concentration of 100 mM. Nω-nitro-L-arginine methyl ester (NAME; Sigma N-5751, St. Louis, MO) was prepared in the same medium at a stock concentration of 500 mM. The stocks were stored at -20°C and working dilutions were prepared fresh for every assay.

Inhibitors of reactive oxygen intermediates (ROIs). The following chemicals were all purchased from Sigma. Ascorbic acid was dissolved in distilled water and butylated hydroxyanisole (BHA) in ethanol to give a 1M stock solution. 2, 2'-bipyridine was prepared in ethanol to yield a 600 mM stock solution. All stock solutions were stored at -20°C. Working dilutions were prepared fresh in the appropriate culture medium for every assay.

Measurement of ROIs by fluorescence. This is a modification of the method described by Rosenkranz et al. (1992). Trophoblasts were cultured in Linbro 96-well plates at $1x10^5$ cells per well in medium alone or containing ROI inhibitors. At the end of the culture period, medium was aspirated, washed gently with 6 volumes of PBS, and 100 µl of Hanks balanced salt solution containing 0.9 mM calcium and 0.4 mM magnesium (HBSS-CM) was added to each well. A 2.5 mg/ml stock solution of 2',7'-dichlorofluorescin-diacetate (DCF-DA; Molecular Probes, Junction City, OR) was prepared in ethanol and added to the wells to give a final concentration of 1 µg/ml. Oxidation of DCF-DA was assessed with a CytoFluor 2350 fluorescence measurement system (Millipore, Bedford, MA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Reagents for sphingomyelin pathway. Sphingomyelinase (SMase) from human placenta were obtained from Sigma (S-5383) and diluted to working concentrations in IMDM/ 10% FCS. The vehicle for the supplied enzyme was a solution of 50% glycerol, 25% potassium phosphate and 0.1% Triton X-100.

Inhibitors for RNA and protein synthesis. Cycloheximide (CHX) was purchased from Sigma and actinomycin D (act D) from Boehringer Mannheim Canada (Laval, Quebec).

E. Materials Specific for Chapter VII:

Reagents. Dexamethasone (Sigma D-4902) was dissolved in ethanol to give a 10 mM stock solution and stored at -20°C. Human recombinant GM-CSF was a generous gift from Sandoz (Basel, Switzerland). CSF-1 was obtained through Dr. P. Ralph (Cetus, Emeryville, CA).

CHAPTER IV

PURIFICATION OF HUMAN TERM PLACENTAL TROPHOBLASTS BY COLUMN-ELIMINATION OF CD9 EXPRESSING CELLS

(Most of the data presented in this chapter has been published as an article entitled "Functional, Long-term Cultures of Human Term Trophoblasts Purified by Column-elimination of CD9 Expressing Cells" by Yui, J., Garcia-Lloret, M., Brown, A.J., Berdan, R.C., Morrish, D.W., Wegmann, T.G. & Guilbert, L.J. in *Placenta* (1994), 15, 231-246.)

A. Introduction

Although valuable information on various aspects of trophoblast has been obtained through the study of organ cultures using human chorionic villi, the complex microstructure of this tissue makes it difficult for investigating the molecular mechanisms involved in the regulation of trophoblast differentiation and/ or function. In order to overcome this problem, a number of methods for the isolation and culture of human trophoblasts have been developed (see Yeger et al., 1989 and references therein). The first step in most methods involves proteolytic disruption of villous microstructure and results in trophoblast preparations with variable contamination of stromal cells such as fibroblasts, endothelial cells and macrophages. These contaminating cells can partly be removed with Percoll density gradients (Kliman et al., 1986) which yield preparations that are 90-95% cytotrophoblasts (Douglas and King, 1989). Although this degree of purity is sufficient for many investigations, a number of studies, such as the examination of trophoblast gene expression by powerful techniques such as polymerase chain reaction (PCR) where as few as 1000

copies of mRNA can be detected (Brady, Barbara and Iscove, 1990), are likely to be hampered by the presence of even low numbers of non-trophoblastic cells. In addition, low levels of non-trophoblastic contaminants may complicate the assessment of the response of trophoblast to drugs, microorganisms or biological response modifiers. Further purification has been achieved with the use of immunomagnetic beads conjugated to various antibodies directed against antigens absent on trophoblasts (Douglas and King, 1989; Morrish et al., 1991; Bischof et al., 1991). However, immunomagnetic adsorption yields trophoblasts that adhere rather poorly in culture (Bischof et al., 1991). In this context, it is noteworthy that the survival, differentiative potential and function of purified trophoblast populations as well as overgrowth by contaminating stromal cells in cultures lasting for greater than one week have not been thoroughly characterized.

In my objective to examine the effects of TNF-α on trophoblasts, it is crucial that we obtain a population of such cells that approaches 100% purity when maintained in culture. Thus, there is a need to investigate methods for trophoblast purification that incorporate the advantages of immuno-elimination but that allow improved yields of cells that remain viable and functional in culture for periods of a week or longer. The approach was to replace immunomagnetic bead separation with the somewhat less expensive and milder procedures offered by antibody-conjugated glass bead columns previously reported for the purification of human and mouse T lymphocytes (Jenkins et al., 1991; Loeffler et al. 1992). Although other antigens probably could be employed for immuno-adsorption, preliminary observations of the protease resistance of the CD9 antigen and its expression on placental fibroblasts but not on trophoblasts in term placental villi have led to continue the development of the anti-CD9 methods previously reported (Morrish et al., 1991). In this

chapter I shall describe experiments showing that placental cells treated with mouse anti-human CD9 monoclonal antibodies and eluted from anti-mouse immunoglobulin conjugated glass bead columns constitute a highly enriched and uniform population of trophoblasts that can be cryopreserved, that survive and function in culture at least two weeks with minimal contamination by mesenchymal cells, and that differentiate to form syncytium-like structures in the presence of epidermal growth factor (EGF).

B. Results

Expression of CD9 in the stroma of term placental villi. Expression of CD9 has been reported on a number of cell types including endothelial cells, lymphocytes and fibroblasts (Andrews, Knowles and Goodfellow, 1981; Forsyth, 1991). However, specific evidence of CD9 expression in term placenta is limited to stromal fibroblasts (Morrish et al., 1991). In order to determine whether other placental cell types also express CD9, frozen sections of human term placenta were immunoperoxidase stained with monoclonal antibody 50H.19. As shown in Figure 4.1a, the entire villous stroma specifically stained positive with this antibody, denoted by a red colour. The trophoblast layer did not react with 50H.19, and was counterstained by hematoxylin to give a blue colour. The control antibody for 50H.19 stained neither the trophoblast layer nor the villous stroma (Figure 4.1b). Parallel studies showed that both placental fibroblasts (Figure 4.1c) and placental macrophages, otherwise known as Hofbauer cells (Figure 4.1d) reacted with the anti-CD9 antibody. Therefore CD9 serves as a useful marker to select against most non-trophoblastic cells within the placental villi.

Removal of contaminating CD9 positive cells by passage through glass bead columns. Suspensions of placental cells prepared by sequential treatment with trypsin-DNase contain from 5 to 10% non-trophoblastic cells (Morrish et al., 1987; Douglas and King, 1989). A similar treatment was used to dissociate cells from placental tissue as trypsin treatment does not remove cell surface CD9 antigen (Morrish et al., 1991). After lysis of red blood cells, CD9 expressing cells were removed from the preparation by first incubating with the

50H.19 antibody, followed by washing, then passing the cells through 1-ml columns of glass beads conjugated with goat anti-mouse Ig (see Chapter III: Materials and Methods). On average 64% of the cells loaded onto the column was retrieved after passage. Flow cytometric analysis using the gates shown in Figure 4.2 revealed that the small population of cells strongly positive for CD9, Leu M5, or W6/32 (HLA Class I) were greatly reduced after column passage (Table 4.1). As observed under the microscope, the placental cell suspension before column passage was heterogeneous with respect to size. There were many vesicular aggregates containing two or more distinct nuclei as well as large and small mononuclear cells. The vesicular aggregates do not register in flow cytometry, thus the before column values in Table 4.1 describe only non-aggregated cells. Both the aggregates and the small cells were retained by the column. The eluted cells consisted of a rather uniform population of large, mononuclear cells that stained positive with the trophoblast specific antibody GB25 (Figure 4.2g). Eluted cells were best used immediately but could be cryopreserved in 10% DMSO in FCS under liquid nitrogen. On thawing, these cells were around 90% viable (by trypan blue exclusion). When plated in medium containing 10% FCS in IMDM at 1×10^5 per 100 µl in 96-microwell, 95% of the viable cells remained adherent after 4 hours of incubation.

Comparison of pre-and post-column cells after one week in culture. When examined 24 hours after plating, the adherent cells demonstrated positive cytokeratin staining manifested in a filamentous pattern under higher resolution fluorescence microscopy (Figure 4.3a) which is characteristic of intermediate filaments of epithelial trophoblasts (Loke et al., 1986). However, it has been reported that contaminating fibroblasts overgrow trophoblast cultures after one week (Yeger et al., 1989). As the small fraction of CD9 and W6/32 positive

cells in post-column cells (Table 4.1) may not accurately reflect true values because the separation between positive and negative signals is small (Figure 4.2), therefore pre- and post-column cells were compared for their propensity to remain free from contamination by non-trophoblastic cells after one week in culture using immunoperoxidase staining, as this method clearly distinguished between positive (stained dark red) and negative cells (no staining) without any intermediate staining (Yui et al., 1994). As examined by immunoperoxidase staining, one week cultures from placental cell preparations before column purification contained CD9 and vimentin positive cells at greater than 2% (Table 4.2). In contrast, one week cultures derived from post-column cells contained no CD9 positive cell out of two thousand cells counted (Table 4.2). Very few cells in 1 week cultures (0.3%) expressed the intermediate filament vimentin, a mesenchymal cell marker (Table 4.2) and >99% expressed the corresponding filament cytokeratin characteristic of epithelial cells (Figure 4.3b). Accordingly, cultures established in 12-well dishes using pre-column cells seeded at lower densities contained fibroblast colonies after 7 days of culture (Figure 4.4a). Such colonies were never seen in parallel cultures established with column purified cells (Figure 4.4b) in three independent experiments performed.

Synthesis of human chorionic gonadotropin (hCG) by column-purified cytotrophoblasts over a week of culture. Function of trophoblasts in culture was assessed by their ability to secrete the pregnancy hormone hCG. Immunohistochemical staining showed that hCG-β was present in greater than 95% of the cells on day 7 of culture (Figure 4.5a). The lymphohematopoietic cytokine GM-CSF enhances hCG production by approximately 2-fold from cultures of unpurified term trophoblasts (Garcia-Lloret, 1991) and therefore was used at 25 ng/ml to optimize hCG production from cultures of column

purified trophoblasts. Levels of hCG in such culture supernatants increased over the first 4 days of culture (Figure 4.5b), thereby indicating that column-purified trophoblasts were functionally active during this period of culture.

Syncytialization of purified trophoblasts after a week of culture. In culture, column-purified trophoblasts form an adherent monolayer in which intercellular boundaries were indistinguishable. In order to determine whether the trophoblasts form syncytium in FCS-supplemented medium, one week cultures were stained with antibody to desmoplakin, components of desmosomes which form intercellular junctions of most epithelia (Garrod and Cowin, 1986). Consistent with the observations of Douglas and King (1990), we observed a pavement-like staining pattern with evenly distributed nuclei (Figure 4.6a) and that mononucleated cells dominated the culture with occasional binucleated cells (Figure 4.7). When epidermal growth factor (EGF) was added to the cultures, it induced a dramatic morphological differentiation in that 50% of the nuclei were found in patches of greater than two nuclei (Figure 4.7). In these syncytialized patches, the nuclei were clustered in groups and often separated by large expanses of cytoplasm (Figure 4.6b). Thus, assessment of the degree of syncytialization by anti-desmoplakin antibodies showed that in the absence of EGF, monolayers of purified trophoblasts after a week in culture even in FCS-containing medium remained mostly non-syncytialized and instead consisted of a patchwork of mononucleated cells.

Purity and viability of long term (2 week) cultures of column purified trophoblasts. If anything, there were fewer vimentin positive cells (0.1 *vs* 0.3%) after two weeks of culture than after 1 week (Table 4.2) and no outgrowth of fibroblast colonies. The DNA content of cultures of purified

trophoblast decreased 35% over a 2 week culture period; however, significant loss occurred only in the first week of culture (Table 4.3). When compared to proliferating cells, trophoblasts showed little proliferative activity on the first day of culture and after a week the values decreased by one-half. In contrast to the DNA content and thymidine uptake, the metabolic capacity (measured by MTT reductive cleavage) and protein content of the cultures were relatively constant over the two week period. These results indicate that the cultures remained viable for at least two weeks.

C. Summary of Results

1. In term placenta, CD9 was expressed on villous stromal cells but not on trophoblasts.
2. Trophoblasts were purified to at least 95% purity by incubating trypsin-derived placental cell preparation with mouse anti-human CD9 antibodies followed by passage through a goat-anti-mouse glass bead column.
3. Placing the cells in culture further improved purity: the cultures were greater than 99% cytokeratin positive and less than 1% vimentin positive after a week in culture.
4. Cells secreted hCG in culture, but failed to differentiate into multinucleated syncytial structures unless EGF was present.
5. The metabolic capacity and protein content of the cultures remained relatively constant during two weeks of culture, but there was a small reduction in the DNA content and thymidine uptake during the first week of culture.

Table 4.1. Comparison of cell yields and cell purity before and after column passage.*

Parameter	Pre-column	Post-column
Cells obtained from 30g of tissue (millions)	146 ± 36	94 ± 21
Percentage of single cells positive for the following markers **:		
CD9	5.3	1.1
Leu M5	7.7	< 0.1
W6/32	14.9	4.7
GB25	-	95.0

* Values shown here are one out of three experiments performed.

**carried out by flow cytometric analysis using the gates indicated in Figure 4.2.

Table 4.2. Comparison of vimentin and CD9 positive cells in pre- and post-column cultures after two weeks.

	Pre-column	Day 7 Post-column	Day 14 Post-column
Percentage of CD9 positive cells	2.3 (2.0-2.8)	< 0.05	ND
p-value*	≤ 0.005		
Percentage of vimentin positive cells	3.3 (1.4-4.9)	0.3 (< 0.05-0.9)	0.1 (<0.05-0.5)
p-value *	≤ 0.05		

Between 1000 to 2000 cells in a total of 5 different fields were counted. Values represent the mean and the range (in brackets) in one out of two experiments performed. Positive cells were scored by counting nuclei whose surrounding cytoplasm demonstrated positive staining.

ND : not done

* analysis by Student t-test

Table 4.3. Viability, DNA and protein content of post-column cells cultured for 2 weeks.

Parameter	Day 1	Day 7	Day 14
MTT readout (O.D. 570-650)	0.335 ± 0.016	0.309 ± 0.012	0.383 ± 0.071
DNA content (ng/well)	201 ± 30	146 ± 16*	130 ± 2*
Protein content (μg/well)	4.5 ± 1.2	5.1 ± 0.8	4.4 ± 0.3
^{3}H-dTR-uptake (cpm)	1740 ± 171	616 ± 23^	662 ± 84^

Values are the mean ± S.D. in triplicates except for ^{3}H-dTR-uptake experiments in which n=5. These values represent one out of three experiments performed.

* $p \leq 0.05$, compared with day 1 values using student t-test.

^ $p \leq 0.001$, compared with day 1 values using student t-test.

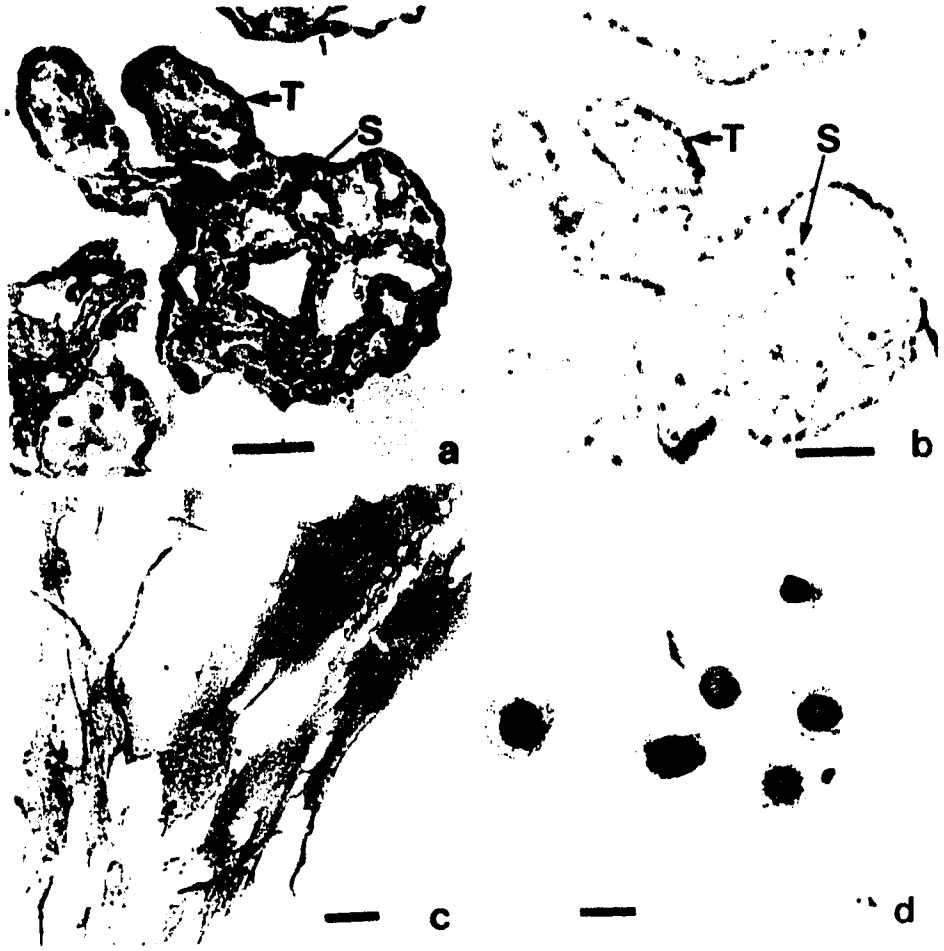


Figure 4.1. Localization of CD9 in the placental villous stroma by immunoperoxidase staining with the anti-CD9 antibody 50H.19. (a) Staining with 50H.19 showing positivity in the stroma (S) and absence of staining in the trophoblast (T). (b) Lack of positive staining with the IgG_{2a} isotype control indicating the reaction in (a) is specific. Bar = 20 μm. Staining with 50.H19 showing positivity in (c) placental fibroblasts and (d) placental macrophages after one week in culture. Bar = 25 μm.

Figure 4.2.

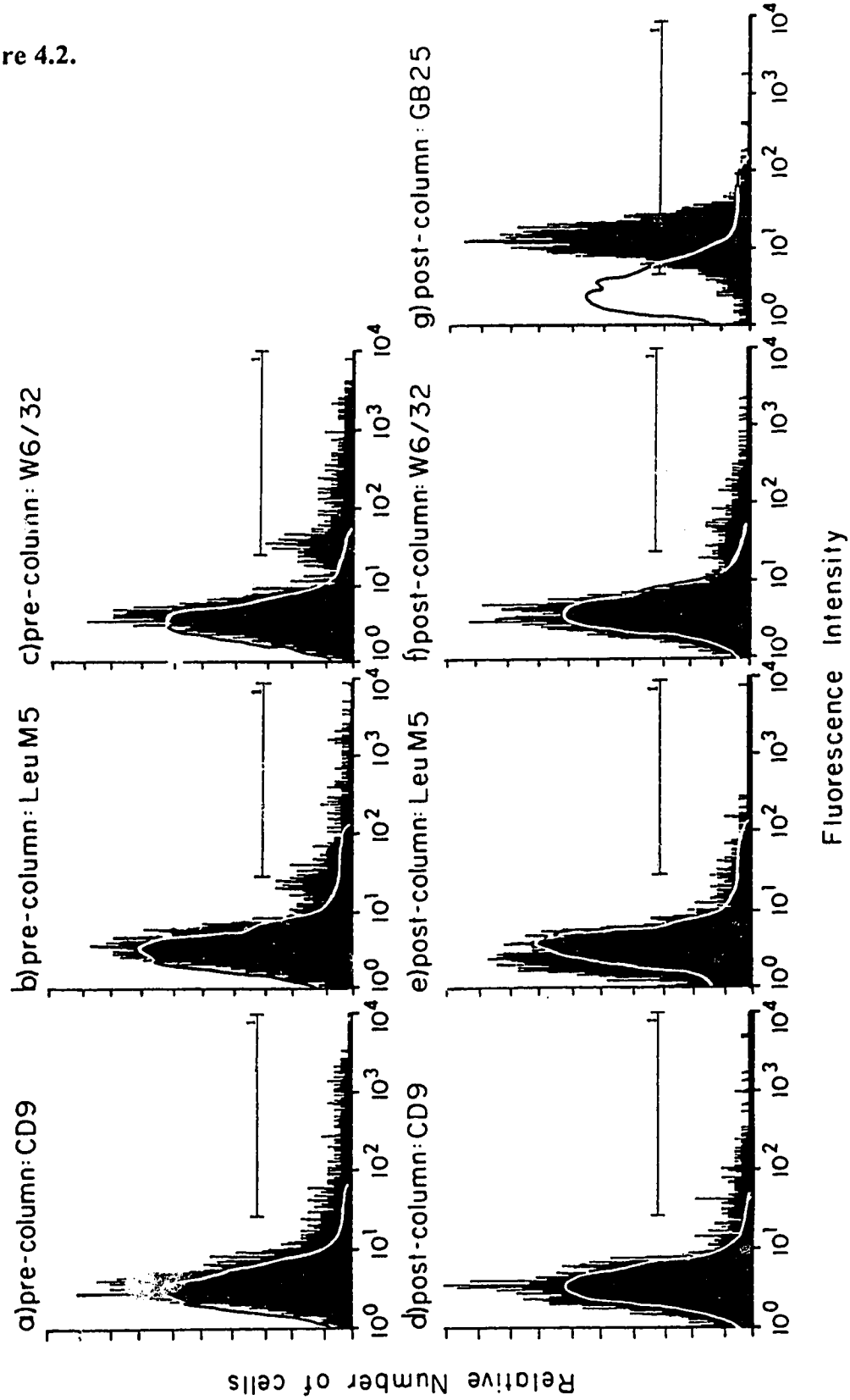
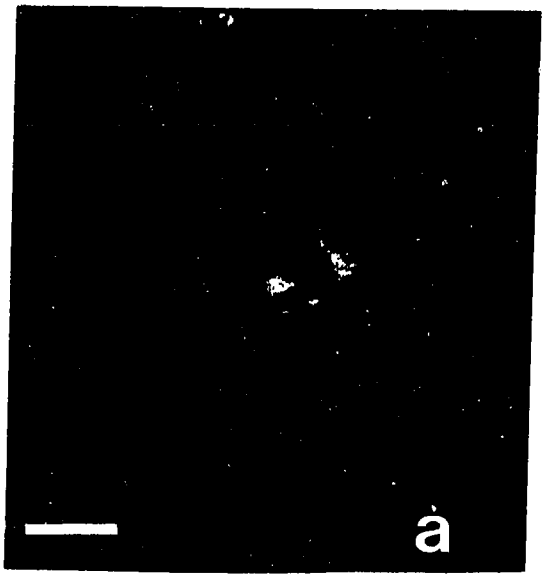

a) pre-column: CD9
b) pre-column: Leu M5
c) pre-column: W6/32
d) post-column: CD9
e) post-column: Leu M5
f) post-column: W6/32
g) post-column: GB25
Relative Number of cells
Fluorescence Intensity

Figure 4.2. Flow cytometric analysis profiles of immunofluorescence-stained placental cells before and after column passage with the following antibodies : (a,b,c) before and (d,e,f,g) after column passage with (a,d) CD9; (b,e) Leu M5 and (c, f) W6/32. Cells after column passage were also stained for (g) GB25. The smooth line represents the isotype controls and the bar refers to the gates used for calculating the values given in Table 4.1.



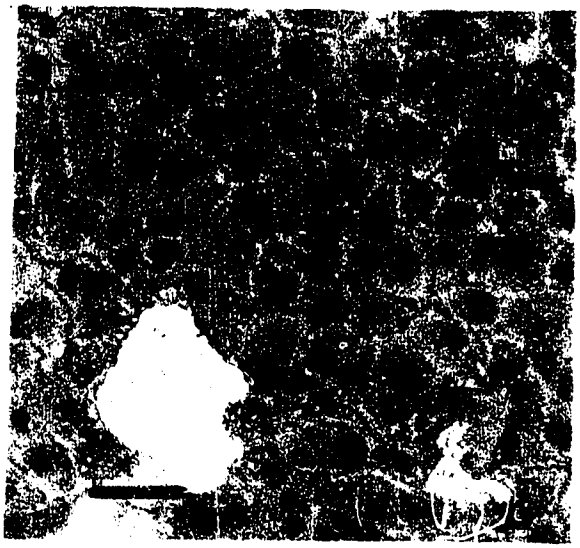


Figure 4.3. Post-column cells cultured for (a) 24 hours on collagen-coated chamber slides stained positive for cytokeratin filaments by immunofluorescence. Bar = 10 μm. (b) Cells cultured for one week in IMDM/10% FCS demonstrated positive staining for cytokeratin by immunohistochemistry. Bar = 50 μm.

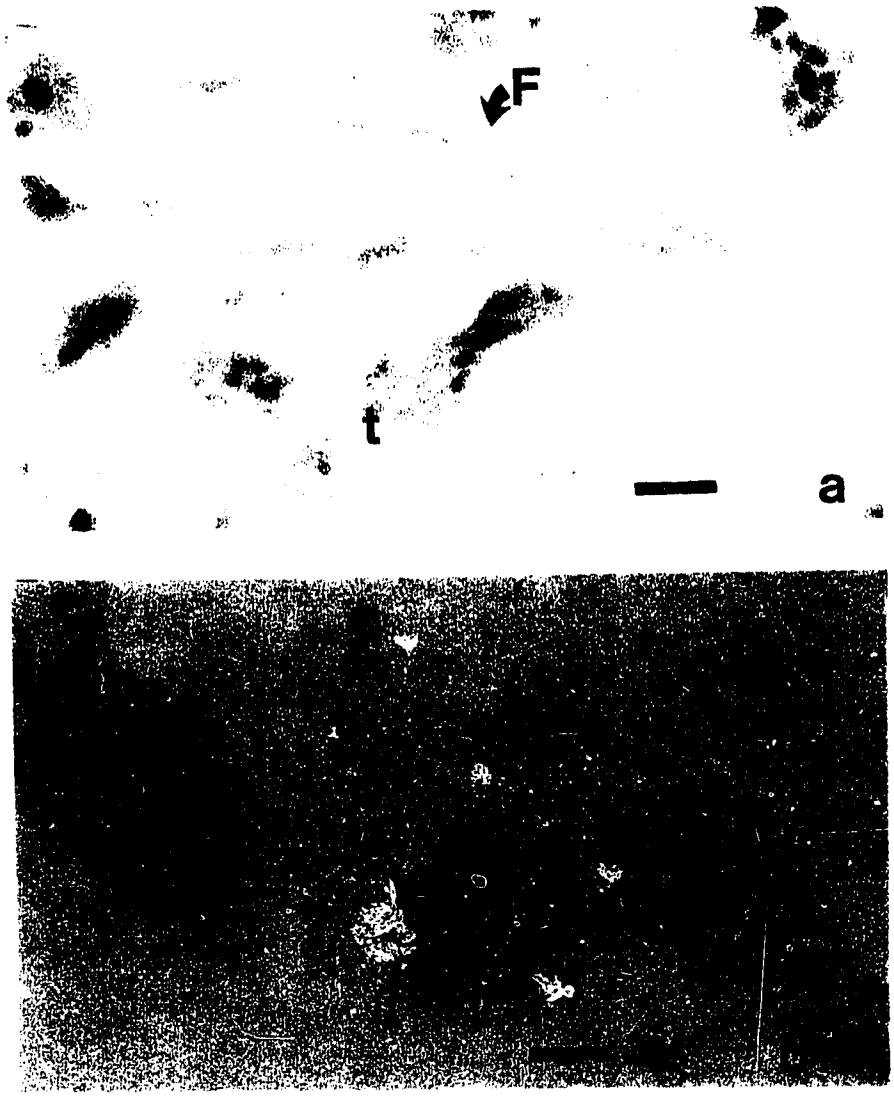


Figure 4.4. Cells (a) before column passage and (b) after column passage were cultured at 1 x 10^6 cells per ml in 12-well Linbro dishes for 7 days and stained with Giemsa. The arrow denotes a fibroblast colony (F) in the midst of two trophoblast clusters (t). Bar = 50 μm.

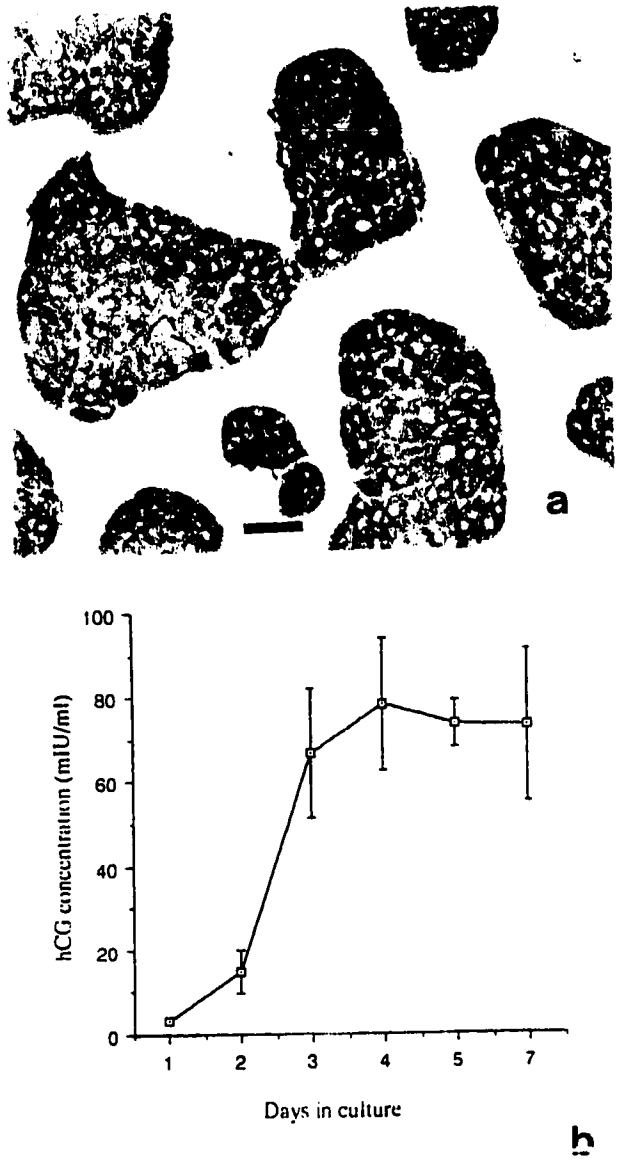


Figure 4.5. Production of hCG by trophoblast cultures demonstrated using (a) immunohistochemical staining (bar = 50 μm) and (b) RIA. Supernatants from column-purified cells cultured at 1 x 10^6 cells/ ml in 10% FCS in IMDM supplemented with 25 ng/ml GM-CSF were removed on designated days, stored frozen at -20°C and assayed for hCG by RIA as outlined in Materials and Methods. The values represent the mean ± S.D. from triplicate wells.

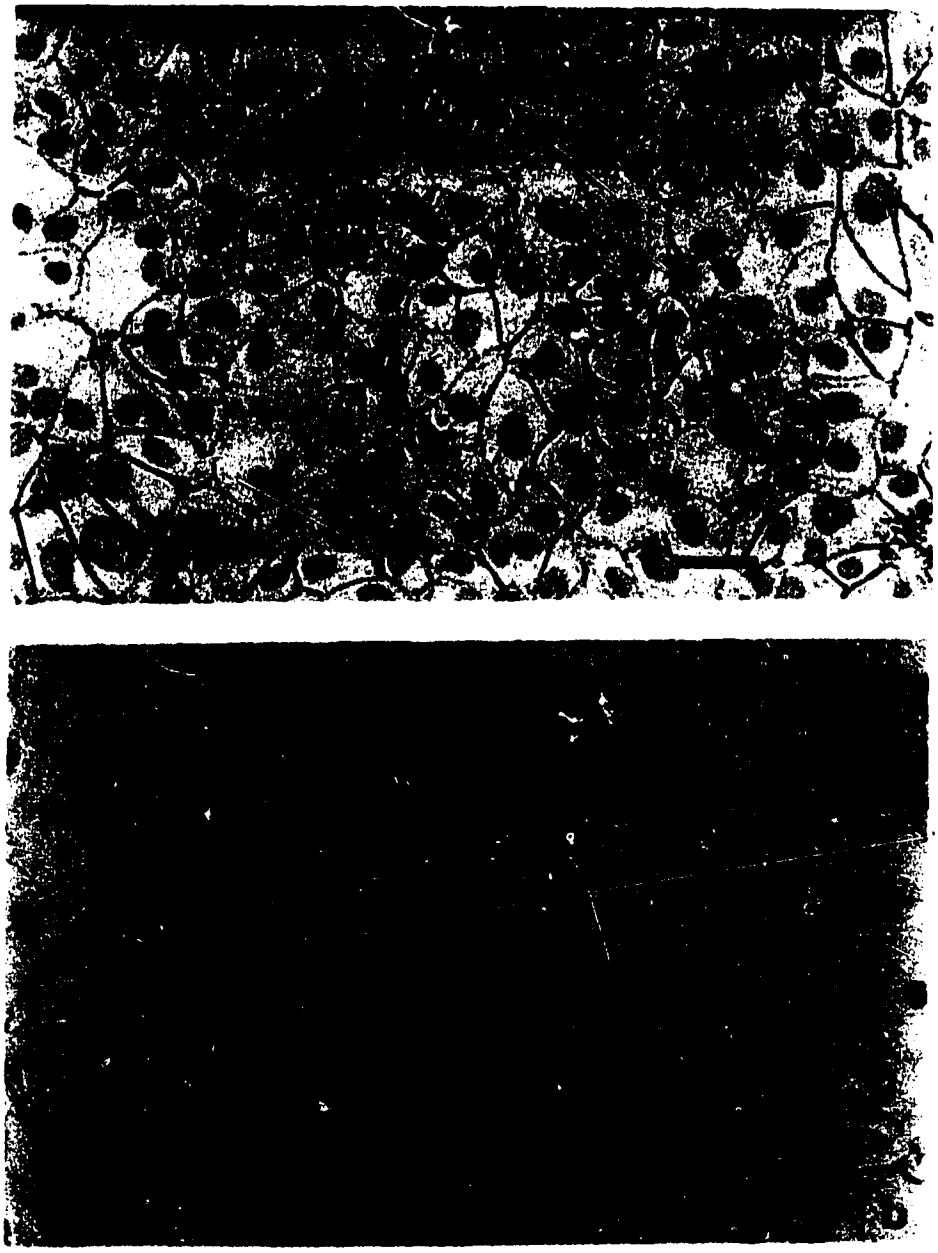


Figure 4.6. Anti-desmoplakin staining of column-purified trophoblasts cultured for a week in (a) IMDM plus 10% FCS and (b) with the addition of EGF (10 ng/ml) showing increased syncytium formation. Bar = 25 μm.

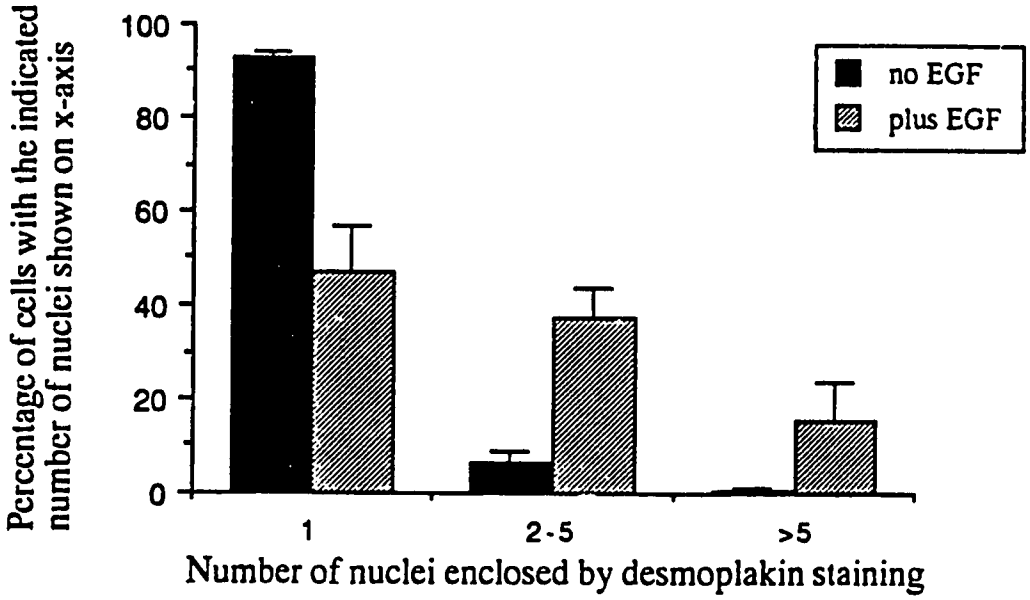


Figure 4.7 Comparison of mono-and multi-nucleated cells in untreated (IMDM plus 10% FCS) and EGF-treated cultures. Cells were stained with anti-desmoplakin antibody after a week in culture, and the number of nuclei within the desmoplakin-delineated cell boundary (see Figure 4.6) was tallied. A total of 1300 nuclei in 10 different fields were counted.

CHAPTER V

TNF-α INDUCES APOPTOSIS IN PRIMARY HUMAN PLACENTAL CYTOTROPHOBLASTS

(Most of the data presented in this chapter has been accepted for publication in *Placenta* as an article entitled "Cytotoxicity of tumor necrosis factor-alpha and gamma-interferon against primary human placental trophoblasts" by Yui, J., Garcia-Lloret, M., Wegmann, T.G. & Guilbert, L.J.)

A. Introduction

There is growing evidence of a bi-directional interaction between the reproductive system and the maternal immune system mediated by lymphohematopoietic cytokines shared by the local uteroplacental and the maternal immune networks (Hunt, 1989; Wegmann, 1990). Studies involving injection of cytokines into pregnant rodents indicate two classes of cytokines in terms of their effects on pregnancy outcome: beneficial and harmful (Chaouat et al., 1990). The latter class is comprised of potent inflammatory cytokines such as IL-2, TNF-α and IFN-γ that regulate cell-mediated immunity. The former class contains not only the macrophage growth factors trophic to placental trophoblasts like CSF-1, GM-CSF and IL-3 but also IL-10, a cytokine defined for its ability to suppress synthesis of pro-inflammatory cytokines (Wegmann et al., 1993).

Injection of TNF-α into pregnant rats at mid-gestation results in placental necrosis and fetal demise (Silen et al., 1989), as do injections of either TNF-α or IFN-γ into pregnant mice (Chaouat et al., 1990). In mouse mating combinations with high rates of spontaneous fetal resorptions, the levels of TNF-α and IFN-γ mRNA expression in placental tissues are greatly elevated

as compared to those from normal mating combinations (Tangri and Ragupathy, 1993). In humans, placental/decidual production of TNF-α in response to local microbial infections has been implicated in the pathogenesis of preterm labor (Casey et al., 1989; Romero et al., 1989). However, the presence of TNF-α and IFN-γ in utero-placental tissues does not always correlate with deleterious effects since TNF-α and IFN-γ are expressed in non-diseased utero-placental tissues (Bulmer et al., 1990; Chen et al., 1991). However, neither the mechanism of the harmful effects of TNF-α and IFN-γ nor the physiological role of their normal placental expression are understood.

Within the villous core, TNF-α and IFN-γ have been reported to be expressed by CTs, ST (Bulmer et al., 1990; Chen et al., 1991) and Hofbauer cells (Berkowitz et al., 1990). Both CTs and ST express receptors for TNF-α and IFN-γ (Austgulen et al., 1992; Peyman and Hammond, 1992; Valente et al., 1992; Yelavarthi and Hunt, 1993) and thus are within the paracrine reach of normal villous expression of these two cytokines. We hypothesize that TNF-α and IFN-γ act on cytotrophoblasts to affect their survival, proliferation and differentiation, and thereby affect placental development and function. It is also important to study the effects of TNF-α on ST since ST is in direct contact with maternal blood, any harmful effects of TNF-α directly on the ST would compromise the integrity of this barrier.

The reported effects of TNF-α and IFN-γ on trophoblasts or trophoblast cell lines are not entirely consistent. High concentrations of TNF-α and IFN-γ inhibit the proliferation of the JEG-3 choriocarcinoma cell line (Berkowitz et al., 1988), yet the JAR line responds mitogenically to TNF-α in an autocrine fashion (Yang et al., 1993). Murine trophoblasts are reported to be resistant to killing by TNF-α (Drake and Head, 1990) whereas TNF-α inhibits DNA

synthesis in rat trophoblast cell lines (Hunt, Atherton and Pace, 1990). Rat labyrinthine trophoblasts, which are the equivalent of human villous trophoblasts, constitute the primary sites of injury after LPS and TNF-α administration (Silen et al., 1989). It has been noted that short term incubation with TNF-α either stimulates (Li et al., 1992) or inhibits (Ohashi et al., 1990) hCG production by semipurified preparations of human trophoblast. However, the effects of TNF-α and IFN-γ on the survival, growth, differentiation or function of defined populations of primary human villous cytotrophoblasts or syncytium have not been investigated. In this study I therefore examined the effects of TNF-α and IFN-γ on cultures that contained fewer than 0.1% contaminating non-epithelial (mesenchymal plus endothelial) cells.

B. Results

TNF-α alone and in combination with IFN-γ disrupts cytotrophoblast monolayers. When seeded at high densities in serum-containing medium, mononucleated cytotrophoblasts adhered after 4 hours and a monolayer of cells was present after 4 days of culture (Figure 5.1a). Within this monolayer, approximately 90% of the cells were mononucleated as determined by staining intercellular boundaries with anti-desmoplakin antibodies. The addition of 10 ng/ml TNF-α after the cells adhered led to the formation of large holes in the monolayer (Figure 5.1b). IFN-γ at 100 U/ml had a minimal effect on the integrity of the monolayer (Figure 5.1c). However, when added with 10 ng/ml TNF-α, IFN-γ enhanced the morphological disruption of the trophoblast monolayer such that only isolated fragments of cellular aggregates were observed after 96 hours of incubation (Figure 5.1d).

The disruptive effect of TNF-α on trophoblasts is concentration and time dependent. The effect of TNF-α and IFN-γ on cytotrophoblasts was initially quantified by monitoring MTT reductive cleavage, a measure of mitochondrial activity that is proportional to the number of viable cells as well as the activation state of the cells (Mosmann, 1983). As shown in Figure 5.2a, TNF-α decreased trophoblast MTT reduction in a dose-dependent manner with maximal effects (approximately 40% loss) reached by 100 ng/ml. When trophoblasts were cultured with high doses of TNF-α (100 ng/ml), prolonged incubation beyond 4 days did not result in a further decrease in MTT reduction, whereas at lower concentrations (10 and 1 ng/ml), trophoblast MTT reductive capacity continued to decrease after 4 days (Figure 5.2b). This

suggests that the disruptive effect of TNF-α on trophoblasts could occur at concentrations below 10 ng/ml provided that degradation of the cytokine could be prevented in culture.

In accordance with observations on morphology, IFN-γ alone had little or no effect on MTT reduction by trophoblasts but enhanced the inhibitory effects of TNF-α at all concentrations tested (Figure 5.2a). In the presence of 100 U/ml of IFN-γ and 100 ng/ml of TNF-α, MTT reduction decreased to 40% of that observed in untreated cultures, although variations in the range from 30% to 60% were sometimes observed when trophoblasts obtained from different placentas were used. Moreover, addition of IFN-γ decreased the half-maximal TNF-α concentration from 3 ng/ml to 1 ng/ml.

In parallel experiments we sought to confirm that the MTT values were a true reflection of the number of viable cells remaining in the culture rather than a change in the cells' activation state after TNF-α and IFN-γ treatments by quantification of the DNA content in individual wells. As seen in Table 5.1, the DNA content of adherent cells under various treatments directly correlated with MTT reduction. Since term trophoblasts hardly p oliferate, the cytokine-induced decrease in MTT reductive capacity was therefore a direct measure of cell loss from the adherent monolayers.

The dynamics of TNF-α expression within placental villi are not known. However, serum TNF levels transiently increase after lipopolysaccharide challenge. Therefore, we asked whether a transient exposure to TNF-α alone or in combination with IFN-γ was sufficient to induce loss of cytotrophoblasts from the cultures. As shown in Figure 5.3, cells exposed to TNF-α or TNF-α plus IFN-γ for 18 hours, then washed and cultured in medium alone for up to 96 hours showed a 21% ($p \leq 0.05$) and 34% ($p \leq 0.001$) decrease, respectively,

in their ability to cleave MTT as compared to control cultures without cytokine treatment.

Trophoblasts cultured in TNF-α and IFN-γ undergo apoptotic death. Since the above observations showed that a combination of TNF-α and IFN-γ enhanced cell loss, we investigated the kinetics of this loss by quantifying the number of cells that appeared in the supernatant within the first 60 hours after cytokine addition. Figure 5.4 shows that the number of cells in the supernatants of control cultures remained relatively constant from 12 to 60 hours of culture. In contrast, cultures treated with TNF-α and IFN-γ contained significantly more non-adherent cells than control cultures ($p \leq 0.05$) after 12 hours and by 60 hours of culture contained 3-fold more non-adherent cells ($p \leq 0.001$) than control cultures. At 60 hours, the non-adherent cells in TNF-α plus IFN-γ treated cultures constituted 43% of the total number of adherent cells present at the initiation of the culture. Under the microscope the majority of these non-adherent cells failed to take up trypan blue and displayed membrane blebbing under EM (R.G. Miller, R..Irvin, M. Garcia-Lloret and L.J. Guilbert, unpublished).

The appearance of non-adherent cells which fail to stain for trypan blue and demonstrate membrane blebbing is suggestive of a form of cell death known as apoptosis (Cohen, 1993). Since TNF-α has been shown to induce apoptosis (Laster, Wood and Gooding, 1988; Obeid et al., 1993), we asked whether other indicators of apoptotic death (DNA nicking, loss of cellular DNA and DNA degradation) were manifested in the trophoblasts of TNF-α and IFN-γ-treated cultures. An early event characteristic of apoptosis is the introduction of nicks in DNA strands by as yet unidentified endonucleases (Arends, Morris and Wyllie, 1990). The enzyme terminal deoxynucleotidyl

transferase (TdT) incorporates nucleotides at sites of DNA nicks, and by inserting biotinylated dUTP, nicks can be visualized via the formation of a biotin-avidin peroxidase complex that in turn can catalyze the local deposition of colored precipitate from suitable substrates (Gavrieli, Sherman and Ben-Sasson, 1992). This method, known as TdT-mediated dUTP-biotin nick end labeling (TUNEL), was used to detect individual cells with DNA strand breaks in control and cytokine-treated cultures within 24 hours of culture (Figure 5.5a and b). After 12 hours of culture, 9% of the nuclei in adherent cells in TNF-α treated cultures contained nicked DNA, and with the addition of IFN-γ, the value increased to 20% (Figure 5.5c). By 24 hours of culture, the proportion of nuclei with nicked DNA is 25% in TNF-α treated cultures and 34% in TNF-α plus IFN-γ treated cultures, as compared to 11% in control cultures (Figure 5.5c).

Since extensive DNA nicks were found in the early stages of cytokine-treated cultures, we determined whether the non-adherent cells in TNF-α and IFN-γ treated cultures had lost cellular DNA by 60 hours of culture. Flow cytometric analysis of the DNA content of non-adherent cells fixed in ethanol in the absence of any cross-linking reagents and stained with propidium iodide showed that approximately 90% of the cells harvested from supernatants of TNF-α and IFN-γ treated cultures (Figure 5.6a) had much less than the original (2n) DNA content of adherent cells harvested 4 hours after plating (Figure 5.6b). There was no significant difference in the forward angle light scatter profiles (a measurement of cell size) between these 2 groups (Figures 5.6c and d) indicating that the same cellular units (and not vesicular fragments) were being analyzed in both groups. Furthermore, by gel electrophoresis, DNA harvested from non-adherent cells in TNF-α and TNF-α plus IFN-γ treated cultures also showed DNA fragmentation (Figure 5.7) typical of apoptotic

death in epithelial cells, during which there is a lack of internucleosomal cleavage to generate a "ladder" pattern of 180-200 base pairs fragments as seen in thymocyte or lymphocyte apoptosis (Oberhammer et al., 1993). Thus, by 60 hours of culture, the large number of non-adherent cells in TNF-α/ IFN-γ treated cultures appeared viable by trypan blue exclusion, contained less than a 2n DNA content as well as fragmented DNA, all of which are characteristics of cell death by apoptosis (Gerschenson and Rotello, 1992; Cohen, 1993).

Susceptibility of EGF-induced syncytium to the cytotoxic effects of TNF-α and IFN-γ. In the above experiments, TNF-α was added to adherent trophoblasts when the cells were mononucleated. We showed previously that EGF added to the cultures induced the formation of multinucleated patches in 50% of the cells (Figure 4.7). In order to examine the effects of TNF-α alone and in combination with IFN-γ as a function of the state of maturation of trophoblast cultures, the cytokines were added to mononucleated trophoblasts (that is, day 0 after adherence), or to trophoblasts which had been cultured in the presence of EGF for a week. As shown in Table 5.2, partially syncytialized cultures were less susceptible to the effects of TNF-α as compared to mononucleated cells at all concentrations tested. As observed with mononucleated cells, the syncytium was also sensitive to the cytotoxic effects of TNF-α and IFN-γ. This can be visualized by the presence of clusters of apoptotic nuclei, marked by the TUNEL technique, in syncytial patches as delineated by desmoplakin staining (Figure 5.8). In some instances, these patches may subsequently detach from the plate and seen as reddish clumps adhering to the monolayer (Figure 5.8).

C. Summary of Results

1. TNF-α induced holes in monolayers of trophoblast cultures which were the result of a loss of trophoblast viability as measured by both metabolic capacity (MTT reduction) and DNA content.
2. IFN-γ alone had no effect, but synergized with TNF-α to enhance the cytotoxic effect.
3. Trophoblasts responded to TNF-α and IFN–γ by undergoing apoptosis, as characterized by DNA nicks after 12 and 24 hours of treatment, and lower DNA content and fragmented DNA in the detached cells harvested after 60 hours in culture.
4. Partially syncytialized cultures formed in the presence of EGF remained sensitive to the cytotoxic effects of TNF-α and IFN-γ, although their response to TNF-α alone waned as compared to mononucleated cultures.
5. Exposure to TNF-α and IFN-γ induced the appearance of apoptotic nuclei clustering within a syncytial unit, indicating that syncytium was also sensitive to the damaging effects of these cytokines.

Table 5.1. Correlation between MTT reductive capacity and DNA content of trophoblasts cultured with TNF-α , IFN-γ and the combination.

Treatments	MTT (O.D.)	% of control	DNA content (ng)	% of control
medium alone	0.345 ± 0.020	100[a]	194 ± 14	100[a]
IFN-γ	0.324 ± 0.009	94[a]	186 ± 7	96[a]
TNF-α	0.242 ± 0.006	70[b]	148 ± 15	76[b]
IFN-γ + TNF-α	0.135 ± 0.013	39[c]	78 ± 11	40[c]

Column-purified trophoblasts were plated at $1x10^5$ cells per well in Linbro 96-well plates and allowed to adhere for 4 hours in IMDM plus 10% FCS. After washing, trophoblasts were cultured for four days in the absence or presence of TNF-α (10 ng/ml) and IFN-γ (100 U/ml) before MTT reductive capacity and DNA content were assessed as described in Materials and Methods.

Values represent the means ± S.D. pooled from 2 independent experiments, using trophoblasts from 2 separate placentas, and each experiment done in triplicates.

Means within a column with no common superscripts differ significantly ($p \leq 0.05$, Tukey's test).

Table 5.2. Susceptibility of EGF-induced syncytium to the effects of TNF-α and IFN-γ.

Treatments	MTT Reduction (% of control)*	
	day 0	EGF-treated
TNF (100 ng/ml)	71 ± 6	94 ± 5
TNF (10 ng/ml)	77 ± 3	98 ± 7
TNF (1 ng/ml)	83 ± 2	102 ± 5
IFN + TNF (100 ng/ml)	57 ± 3	75 ± 7
IFN + TNF (10 ng/ml)	70 ± 4	79 ± 7
IFN + TNF (1 ng/ml)	82 ± 3	100 ± 4

Column-purified trophoblasts were plated at $1x10^5$ cells per well in Linbro 96-well plates and allowed to adhere for 4 hours in IMDM plus 10% FCS. After washing, trophoblasts were cultured in EGF (10 ng/ml) for 7 days to induce partial syncytium formation before the addition of TNF-α alone or in combination with IFN-γ. For day 0 cells, these cytokines were added after the routine 4 hour adherence step. MTT assay was performed after 96 hours in culture. * Percentage was calculated based on MTT readout of control cultures in medium alone without cytokine additions, for day 0 cultures, the value was 0.518; for EGF-treated cultures, the value was 0.361. Percentages represented the mean ± SD of 6 samples, pooled from 2 separate experiments, each performed in triplicates.

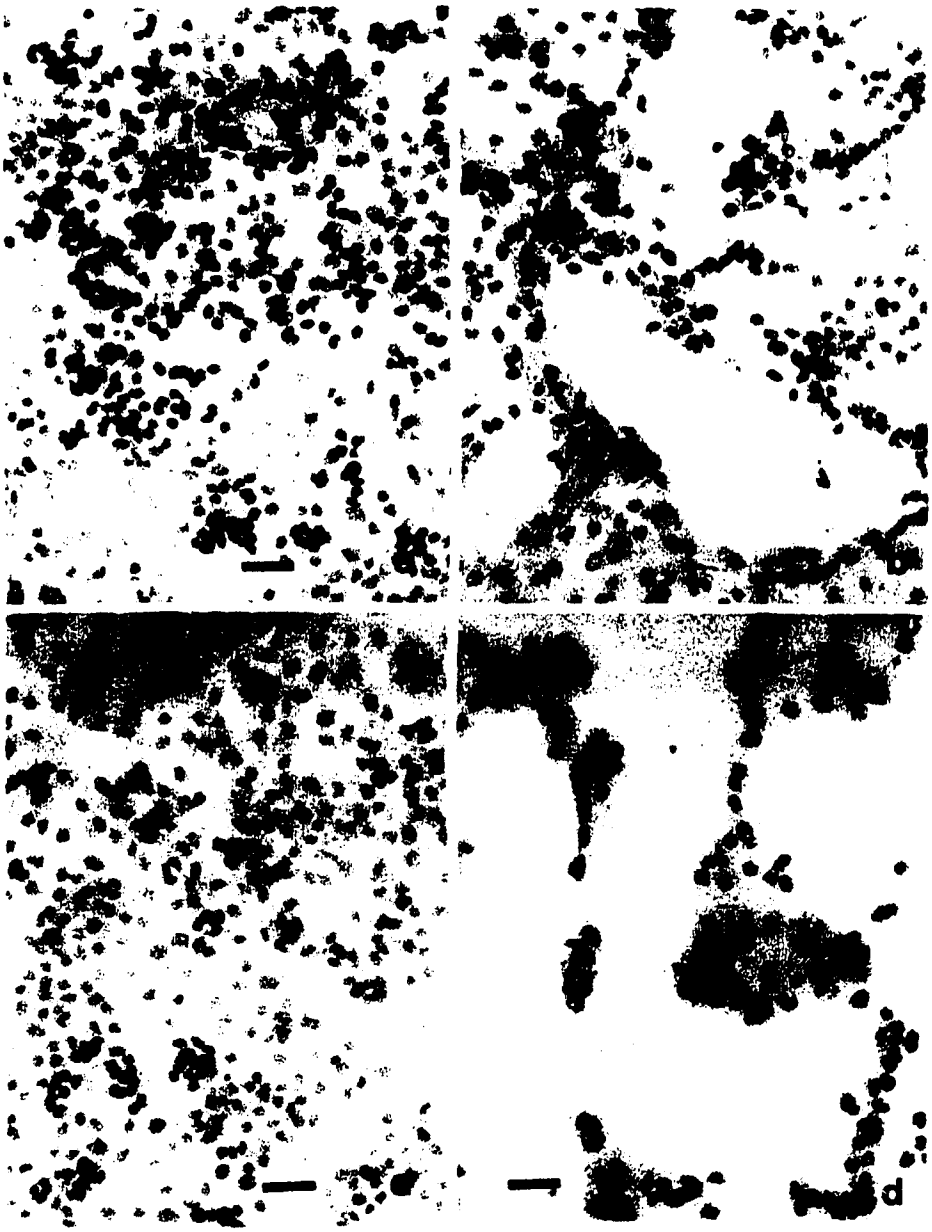
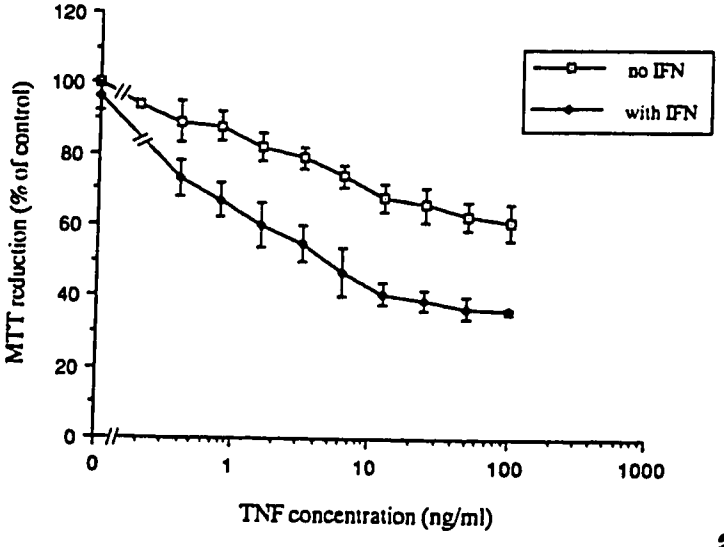
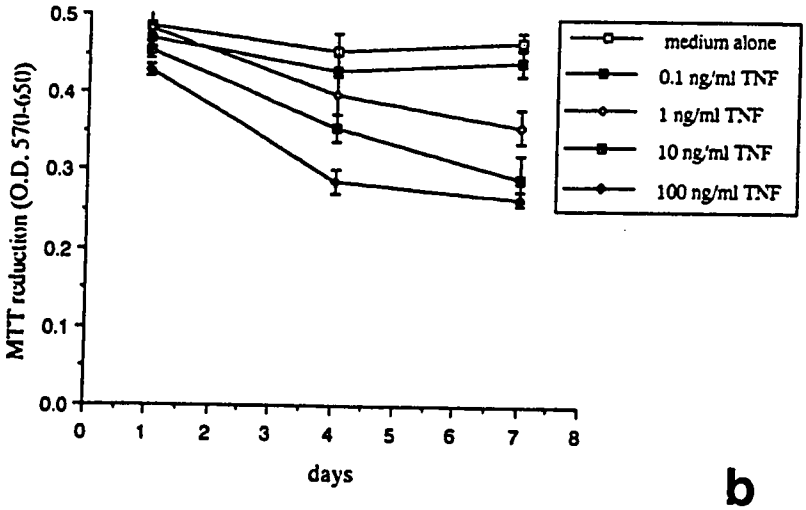


Figure 5.1. TNF-α alone and in combination with IFN-γ disrupts trophoblast monolayers. Column-purified trophoblasts were cultured for 4 days (a) in medium (10% FCS in IMDM) alone; (b) with 10 ng/ml TNF-α; (c) with 100 U/ml IFN-γ and (d) with a combination of 10 ng/ml TNF-α and 100 U/ml IFN-γ. Stained with Wright-Giemsa. Similar observations were noted in at least five independent experiments. Bar = 50 μm.

Figure 5.2.


120
100
80
60
40
20
0
MTT reduction (% of control)
0
1
10
100
1000
TNF concentration (ng/ml)
no IFN
with IFN

a


0.5
0.4
0.3
0.2
0.1
0.0
MTT reduction (O.D. 570-650)
0
1
2
3
4
5
6
7
8
days
medium alone
0.1 ng/ml TNF
1 ng/ml TNF
10 ng/ml TNF
100 ng/ml TNF

b

Figure 5.2. The effects of TNF-α on trophoblasts as a function of (a) concentration and (b) time. Column-purified trophoblasts were plated at $1x10^5$ cells per well in Linbro 96-well plates and allowed to adhere for 4 hours in IMDM plus 10% FCS. After washing, TNF-α at arious concentrations was added to the adherent trophoblasts for the indicated incubation period, then wells were washed once, replenished with 100 μl of IMDM plus 10% FCS and the MTT assay was carried out as described in Materials and Methods. (a) Titration curve of TNF-α with and without 100 U/ml IFN-γ on trophoblast cells cultured for 4 days. The values are expressed as a percentage of control in medium without TNF-α addition and represent the mean of six wells ± S.D pooled from two separate experiments, using two different batches of placental trophoblasts and each experiment performed in triplicates. (b) MTT values were determined on days 1, 4, and 7 of culture. For the day 7 cultures, medium with or without TNF was changed once on day 4. The values represent the mean of triplicate wells ± S.D. The experiment was repeated twice with similar results.

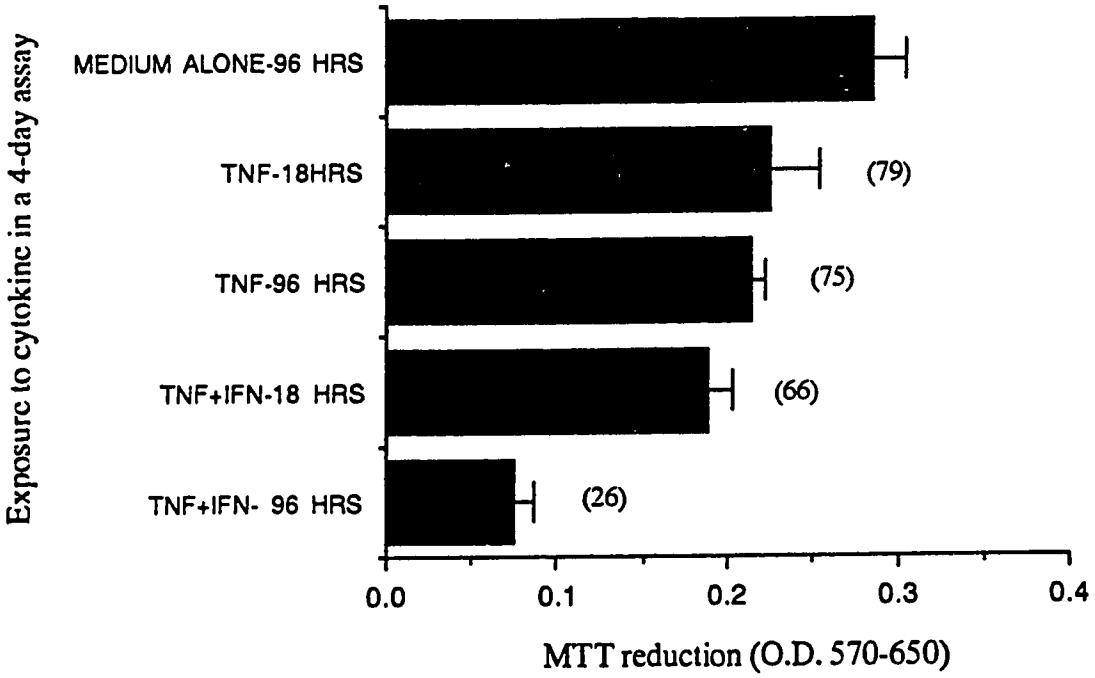


Figure 5.3. Effect of transient TNF-α exposure on cell loss from trophoblast cultures. Column-purified trophoblasts were plated at $1x10^5$ cells per well in Linbro 96-well plates and after adherence and washing away non-adherent cells, trophoblasts were cultured in medium alone, TNF-α (10 ng/ml) or TNF-α (10 ng/ml) plus IFN-γ (100 U/ml) for 18 hours, then washed 3 times in warm medium. Either fresh medium alone (10% FCS in IMDM) or medium supplemented with cytokines was added to parallel wells and the incubation was continued to a total of four days before performing an MTT assay. Numbers in brackets represent the readout as a percentage of the value in medium alone. The values represent the mean of quadruplicate wells ± S.D. in one out of three independent experiments, all of which yielded similar results.

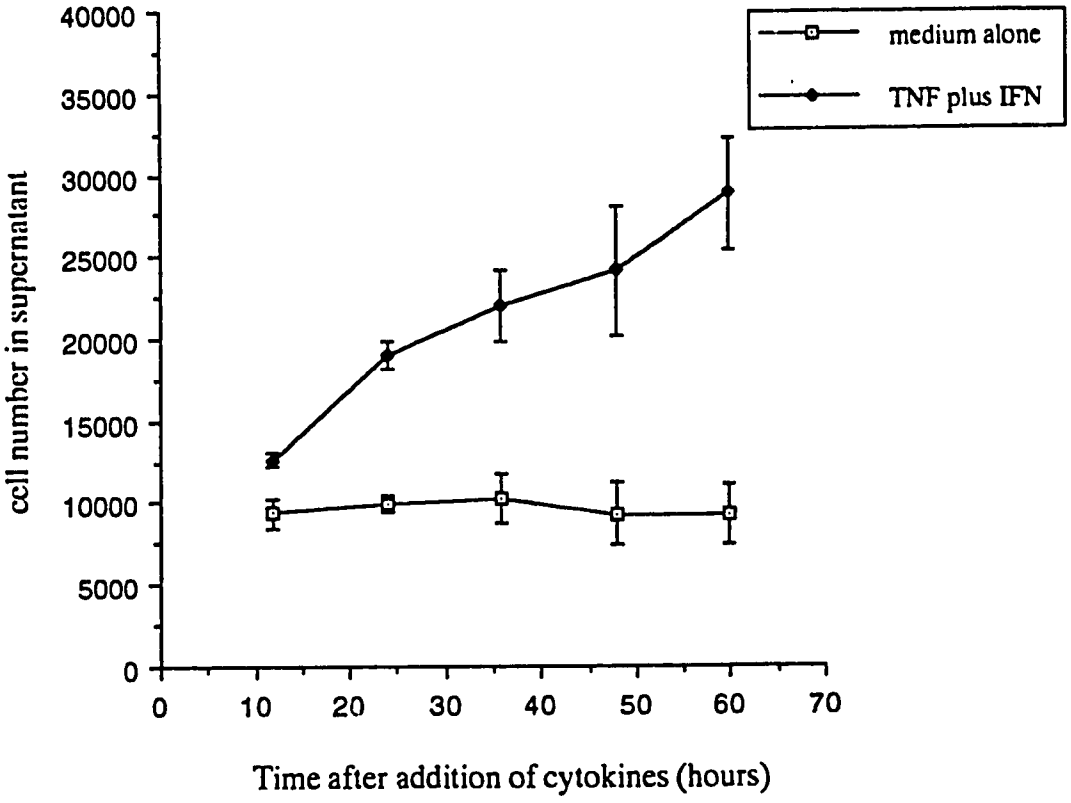


Figure 5.4. Comparison of numbers of non-adherent cells in trophoblast cultures containing medium alone or TNF-α (10 ng/ml) and IFN-γ (100 U/ml). Column-purified trophoblasts were plated at $1x10^5$ cells per well in Linbro 96-well plates and allowed to adhere for 4 hours in IMDM plus 10% FCS. At the time points indicated on the abscissa, cells in supernatants were pelleted by centrifugation at 200g for 5 minutes, stained with trypan blue and counted in a hemacytometer. The values represent the mean ± standard error pooled from two independent experiments, each with triplicate wells.

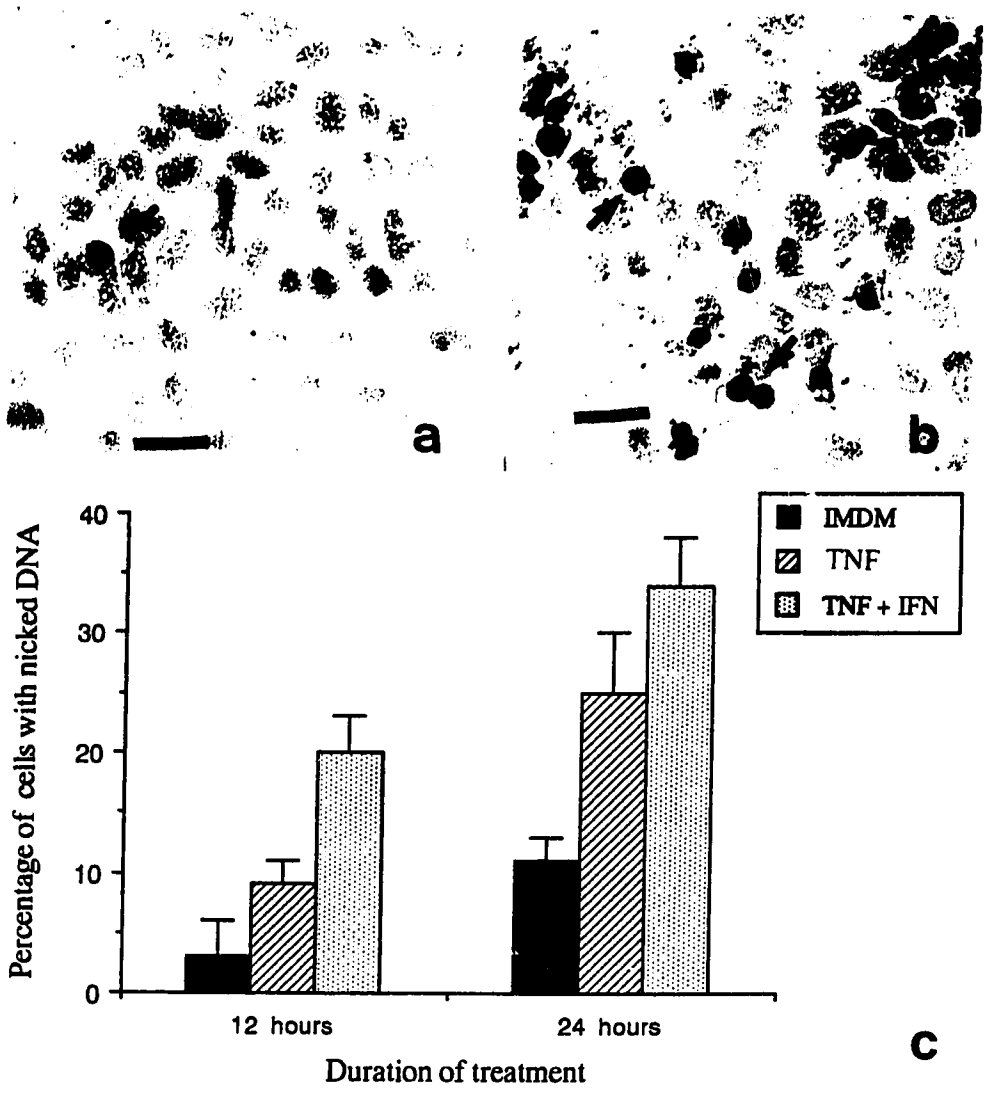


Figure 5.5. Early morphological changes induced by TNF-α and IFN-γ in trophoblast cultures. After a 4 hour adherence step in IMDM plus 10% FCS, column-purified trophoblasts were cultured in the (a) absence or (b) presence of TNF-α (10 ng/ml) and IFN-γ (100 U/ml) for 12 hours and TUNEL was performed to reveal nuclei containing nicked DNA which stained red (denoted by arrows) whereas normal nuclei were counterstained by hematoxylin to give a light purple colour. Bar = 25 μm. (c) Percentage of cells containing nuclei with nicked DNA as determined by TUNEL after 12 and 24 hours of culturing in normal medium, medium containing TNF-α and TNF-α plus IFN-γ. Values represent the mean ± S.D. of five random fields, each having between 100-140 nuclei.

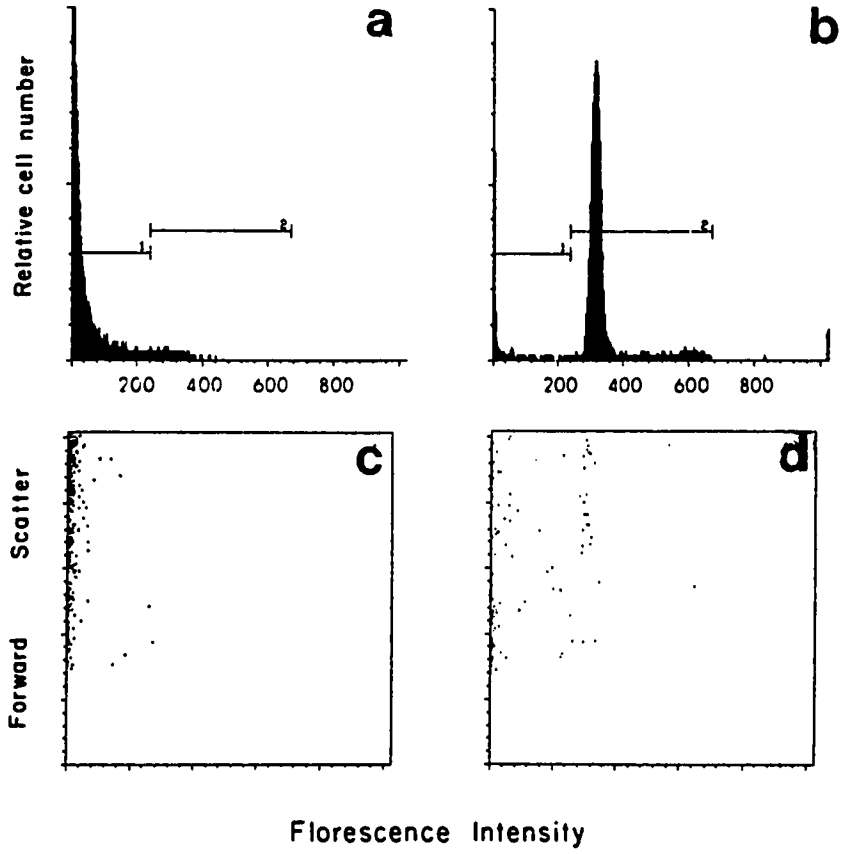


Figure 5.6. Flow cytometric analysis of DNA content on (a) non-adherent cells in TNF-α (10 ng/ml) plus IFN-γ (100 U/ml) treated cultures for 60 hours compared to (b) adherent mononucleated trophoblasts before treatment with the above cytokines. Cells were fixed in ethanol and stained with propidium iodide as described in Materials and Methods. The gates indicated were used to calculate the fraction of cells containing 2n and greater DNA content (gate 2) or less than 2n content (gate 1). Forward angle light scatter profiles of (c) cytokine-treated trophoblasts versus (d) mononucleated trophoblasts showed similar cell sizes in both analysis. This experiment was repeated three times with similar results.

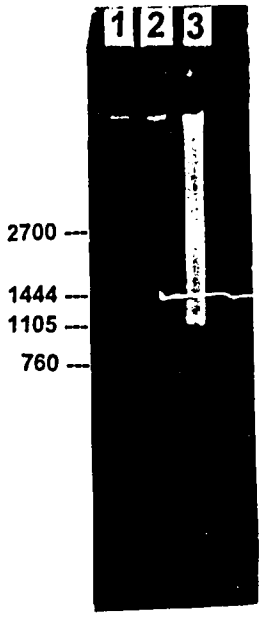


Figure 5.7. DNA fragmentation in cultured trophoblasts as visualized by gel electrophoresis. Column-purified trophoblasts were plated at $1x10^6$ cells per well in Costar 6-well plates and after adherence and washing away non-adherent cells, trophoblasts were cultured in either medium alone, TNF-α (10 ng/ml) or TNF-α plus IFN-γ (100 U/ml). DNA was harvested from the non-adherent fraction of trophoblast cultures at 60 hours in medium alone (lane 1), TNF-α (lane 2) and TNF-α plus IFN-γ (lane 3). A constant fraction of each harvest was run on a 1% agarose gel and stained with ethidium bromide as described in Materials and Methods. Molecular weight markers are in base pairs. This experiment was repeated six times with similar results.

Figure 5.8. Demonstration of apoptosis in syncytium exposed to TNF-α and IFN-γ. Column-purified trophoblasts were plated at $1x10^5$ cells per well in Linbro 96-well plates and after adherence and washing away non-adherent cells, were cultured in EGF (10 ng/ml) for a week to induce syncytium formation. After extensive washing to remove residual EGF, TNF-α (50 ng/ml) and IFN-γ (100 U/ml) were added to the wells and cultured for 18 hours before performing TUNEL (to reveal apoptotic nuclei) followed by desmoplakin staining (to delineate intercellular boundaries) as described in Materials and Methods. Closed arrow: apoptotic nuclei within a syncytial unit. Open arrow: a patch of what appears to be apoptotic syncytium detached from the plate. Bar = 25 μm.

CHAPTER VI

MECHANISMS ASSOCIATED WITH TNF-α-INDUCED CYTOTOXICITY IN CULTURED CYTOTROPHOBLASTS.

A. Introduction

It is generally accepted that TNF-α initiates its biological actions via binding to cell surface receptors. Two high affinity TNF-Rs of molecular masses 55 kd (p55 or TNF-RI) and 75 kd (p75 or TNF-RII) have been cloned and characterized structurally (Loetscher et al., 1990; Smith et al., 1990). Their extracellular domains share 28% homology and together with a number of cell surface receptors such as the nerve growth factor receptor, Fas antigen, CD40, CD27, CD30 and OX40, they are grouped into the TNF receptor superfamily in which each individual member is characterized by cysteine-rich repeats in its extracellular domains (Smith, Farrah and Goodwin, 1994; Beutler and Van Huffel, 1994).

The intracellular domains of p55 and p75 lack any sequence similarity, suggesting that they may trigger distinct signaling pathways. In mice, the proposal that p55 and p75 mediate largely non-redundant signals is supported by antibody blocking studies and the generation of receptor knockout mice. Thus, murine p55 is involved in cytotoxicity, induction of manganous superoxide dismustase mRNA (Tartaglia et al., 1991), antiviral responses (Wong et al., 1992), endotoxic shock and the clearance of intracellular pathogens (Pfeffer et al., 1993; Rothe et al., 1993). On the other hand, murine p75 transduces signals for the proliferation of primary thymocytes and cytotoxic T-lymphocytes (Tartaglia et al., 1991). In humans, however, most TNF-mediated biological activities and signal transduction cascades are

attributed to the p55 TNF-R, whereas the function of the p75 TNF-R remains obscure (Greenblatt and Elias, 1992; Wiegmann et al., 1992; Loetscher et al., 1993; Tartaglia et al., 1993). Nonetheless, in certain cell lines, both p55 and p75 are implicated in the induction of cellular cytotoxicity (Shalaby et al., 1990; Heller et al., 1992). Recently, it has been reported that p55 alone is sufficient to trigger cell death by necrosis, whereas apoptosis requires signaling through both TNF receptors (Higuchi and Aggarwal, 1994).

Dissection of the biochemical signaling processes of TNF is further complicated by the fact that the primary structures of the two receptors do not reveal any significant consensus sequences to suggest the association with a particular classical signaling pathway, although a short cytoplasmic stretch of the p55 TNF-R bears homology with the Fas antigen known to induce apoptosis (Itoh et al., 1991). A number of intracellular secondary messengers have been reported to be associated with TNF-α-mediated apoptosis. These include the generation of reactive nitrogen intermediates (RNIs), reactive oxygen intermediates (ROIs), and the hydrolysis of sphingomyelin.

RNIs include nitric oxide (NO) and its interrelated redox forms: the nitrosonium cation (NO^+) and nitroxyl anion (NO^-), with NO being the most widely studied among the three (Stamler, Singel and Loscalzo, 1992). NO is freely diffusible across cell membranes and is synthesized by various cells including neurons (Bredt and Snyder, 1990), macrophages (Lowenstein et al., 1992; Xie et al., 1992), endothelial cells (Janssens et al., 1992; Marsden et al., 1992), neutrophils (Wright et al., 1989) and hepatocytes (Curran et al., 1989; Geller et al., 1993). The synthesis of NO is dependent on the enzyme NO synthase, which converts L-arginine into NO and L-citrulline. There are at least three different isoforms of NO synthase: one is calcium- and calmodulin-dependent and is constitutively expressed in endothelial cells and neurons

(Kolb and Kolb-Bachofen, 1992). The second form is independent of calcium or calmodulin control. Its expression is induced in murine macrophages by IFN-γ or TNF-α plus IFN-γ (Deng et al., 1993). The third form has been cloned from human hepatocytes, is inducible by TNF-α, IL-1 and IFN-γ but its activity is regulated by calcium and calmodulin (Geller et al., 1993). Due to their different properties, it is not surprising that analogues of L-arginine such as N^G-monomethyl-L-arginine (NMAA) or N^G-nitro-L-arginine methyl ester (NAME) exhibit selectivity in blocking the activity of different classes of NO synthase (Lambert et al., 1991).

The ability of activated macrophages to kill tumors and a number of fungal, bacterial and protozoan parasites is partly attributed to the enhanced production of NO by these cells after treatment with IL-1, TNF-α, and IFN-γ (Stuehr and Nathan, 1989; Lin and Chadee, 1992). Interestingly, activated macrophages undergo apoptosis that is mediated by NO (Albina et al., 1993). NO is also cytotoxic to myelin-producing oligodendrocytes (Merrill et al., 1993) and impairs the insulin secretion of pancreatic β-cells (Corbett et al., 1993).

NO causes cell death by forming complexes with nonheme iron containing enzymes, thereby interfering with crucial cellular processes such as DNA replication, mitochondrial respiration, and/or the Krebs cycle. In combination with superoxide, an oxygen free radical, NO gives rise to peroxynitrite which disrupts cell membranes via lipid peroxidation (Larrick and Wright, 1990).

Exposure to TNF-α also induces the formation of ROIs which include hydrogen peroxide, hypochlorite anion, superoxide anion and hydroxyl free radical, the last being the most damaging and toxic of the ROIs (Camussi et al., 1991). These highly reactive species can be formed in the mitochondrial

respiratory chain, by the action of xanthine oxidase in peroxisomes, during the respiratory burst of neutrophils and macrophages and as byproducts of arachidonate metabolism initiated by PLA_2 activation (Larrick and Wright, 1990; Pruzanski and Vadas, 1991; Janssen et al., 1993). These species are detrimental to cell survival because of their extreme instability; thus, they react with susceptible molecules in their vicinity, causing lipid peroxidation, oxidation of proteins and DNA alterations including single and double strand breaks and the crosslinking of DNA (Janssen et al., 1993).

TNF-α upregulates the production of ROIs in cell lines sensitive to the toxic effects of TNF-α but not in resistant cells. Antioxidants and iron chelators, the latter of which bind the cation to prevent it from catalyzing the Fenton reaction which in turn generates hydroxyl radicals, are also effective in inhibiting TNF-α induced killing (Matthews et al., 1987; Yamauchi et al., 1989; Schulze-Osthoff et al., 1992). This applies not only to transformed cells, but also to primary rat pancreatic β-cells which are destroyed by the combination of TNF-α and IFN-γ (Sumoski, Baquerizo and Rabinovitch, 1989; Rabinovitch et al., 1992). In addition to chemical antioxidants which act as scavengers of oxygen free radicals, cells are capable of generating their own antioxidant enzymes. Glutathione and its related cellular sulfhydryl proteins are involved in the natural detoxification of ROIs; a decrease in the levels of these proteins enhances the ability of TNF-α to cause tumor necrosis *in vivo* (Zimmerman et al., 1989). Other enzymes such as catalase and glutathione peroxidase, which break down hydrogen peroxide, and the mitochondrial superoxide scavenging enzyme manganous superoxide dismutase have all been shown to protect cells against the cytotoxic effects of TNF-α (Zimmerman et al., 1989; Wong et al., 1989; Janssen et al., 1993). On the other hand, reagents that inhibit superoxide dismutases (for example, nitroprusside) or which

irreversibly bind the free sulfhydryl groups of glutathione (for example, diethylmaleate) enhance the cytotoxic effects of TNF-α (Zimmerman et al., 1989; Schulze-Osthoff et al., 1992; Kane et al., 1993). Recently, it has also been shown that TNF-α also downregulates glutathione levels in monocytic U937 cells and that this event triggers apoptosis (Malorni et al., 1993).

A recently discovered pathway by which TNF-α can mediate apoptosis is via the generation of ceramide as a result of sphingomyelin hydrolysis by a neutral sphingomyelinase (SMase; Kolesnick and Golde, 1994). This process is similar to the breakdown of phosphatidyl-inositol by phospholipase C (PLC). Both signaling pathways utilize components of the lipid membranes and generate second messengers, ceramide and diacylglycerol (DAG), respectively, that partition across the plasma membrane lipid bilayer. Like DAG, ceramide also activates kinases, but not protein kinase C (Kolesnick and Golde, 1994). In cell lines that exhibit sensitivity towards TNF-induced cytotoxicity, such as the monoblastic U937 and the promyelocytic HL60, as well as those of non-hematopoietic origin such as the fibroblastic L929 and WEHI-1640, the addition of ceramide analogues or exogenous sphingomyelinase (SMase) increases intracellular ceramide levels and leads to cell death via apoptosis, mimicking the effects of TNF on these cells (Obeid et al., 1993; Jarvis et al., 1994). Although the mechanisms and intermediates associated with this novel signaling pathway remain obscure, it is proposed that TNF activates a plasma membrane neutral SMase which converts sphingomyelin to phosphocholine and ceramide (Dressler, Mathias and Kolesnick, 1992). The latter serves as a second messenger, activating downstream protein kinases and proteases, and these signals may eventually culminate in the activation of calcium-dependent endonucleases involved in fragmenting DNA into nucleosome-sized fragments (Carson and Riberio, 1993).

In the previous study, we demonstrated that TNF-α induced apoptosis in cultured trophoblasts. In this chapter, we investigate the participation of the two TNF-Rs, and the potential involvement of RNIs, ROIs and ceramide as signaling intermediates along the pathway which result in the apoptotic death of cultured trophoblasts upon exposure to TNF-α.

B. Results

Involvement of the p55 and p75 TNF-Rs in signal transduction of TNF-α-mediated cytotoxicity. To delineate the roles of p55 and p75 in TNF-α-induced cytotoxicity in cultured trophoblasts, I tested two different anti-TNF-R antibodies, htr-9 and utr-1, for their influence on the cytotoxic effect. The former (htr-9) was raised against p55 and behaves as an agonist whereas the latter (utr-1) acts as an antagonistic antibody against p75 (Espevik et al., 1990; Yanaga and Watson, 1992). As shown in Figure 6.1, htr-9 alone and in combination with IFN-γ was able to reproduce the TNF-α-induced cytotoxicity on trophoblasts, although not as effective as 10 ng/ml of TNF-α. On the other hand, utr-1, as an antagonist, failed to block the cytotoxic action of TNF-α and IFN-γ on trophoblasts (Table 6.1).

In addition, I also tested two TNF-α mutants which bind selectively to the p55 or p75 TNF-Rs. R32W-S86T carries a double mutation at positions 32 and 86 and binds selectively to p55 whereas D143N-A145R harbours mutations at positions 143 and 145 and binds to p75 but not to p55. The p55-specific TNF-α mutant (R32W-S86T) caused 30% and 76% loss of cell viability when used alone or in combination with IFN-γ respectively (Figure 6.2). This was significantly ($p \leq 0.05$) less effective (14% less) when compared to wild type TNF-α used at the same concentration. On the other hand, cytotoxicity was not observed with p75-specific TNF-α mutant (D143N-A145R) and its presence did not increase the cytotoxic activity of p55-specific mutant (R32W-S86T) at the concentration tested (Figure 6.2). These observations suggest that p55 is the major TNF receptor involved in signal transduction of cytotoxicity in trophoblasts.

NO is not involved in TNF-a-mediated cytotoxicity of trophoblasts.

The p55 TNF-R has been associated with the induction of NO synthase (Tartaglia et al., 1993). To investigate whether NO production is involved as a second messenger leading to the apoptotic death of cultured trophoblasts induced by TNF-α, cells were cultured in arginine-free RPMI medium reconstituted with increasing concentrations of L-arginine. Since NO is derived from arginine by the enzyme NO synthase, the use of arginine-deficient medium should cause a marked reduction in NO production by these cells. We observed that even without an exogenous supply of arginine, cells remained fully sensitive to the effects of TNF-α and TNF-α and IFN-γ (Table 6.2). The degree of killing remained relatively unchanged despite increasing the exogenous supply of L-arginine from 20 to 200 μg/ml. This range encompasses the arginine levels found in some of the most commonly used culture medium such as IMDM, DMEM or RPMI. On the other hand, extremely high doses of arginine were toxic to the cells (Table 6.2) probably by blocking the oxidative respiratory chain (Albina et al., 1993).

We also used analogues of L-arginine as competitive inhibitors of NO synthase to block the generation of NO. The concentrations used for NMMA (1 mM) and NAME (5mM) were chosen on the basis of their ability to effectively suppress NO synthase in macrophages, neural and endothelial cells (Lambert et al., 1991; Merrill et al., 1993). In trophoblasts, both NMMA and NAME were ineffective in inhibiting the cytotoxic effects induced by TNF-α and IFN-γ, although NMMA, but not NAME, was slightly effective ($p \leq 0.05$) in inhibiting cytotoxicity induced by TNF-α alone (Table 6.3). The above results are in accord with the low levels of NO from the supernatants of trophoblast cultures stimulated with TNF-α and IFN-γ as measured by the Griess assay. All levels were below the detection limit (about 2 μM), which were low when compared

to those obtained from the supernatants of similar numbers of activated macrophages (range from 10-40 μM; Merrill et al., 1993). Taken together, these data strongly indicate that NO is not an important intermediate in the cytotoxic pathway.

ROIs are not involved in TNF-α-induced cytotoxicity of trophoblasts. In several cell systems sensitive to its cytotoxic effects, TNF-α is known to upregulate the production of ROIs and inhibitors of ROIs block TNF-induced cytotoxicity (Matthews et al., 1987; Rabinovitch et al., 1992). We found that although trophoblasts were killed in the presence of TNF-α alone or in synergy with IFN-γ, these cytokines did not increase the cellular production of ROIs. If anything, the production of these ROIs was decreased (Table 6.4). In addition, inhibitors of ROIs such as ascorbic acid and BHA which were effective in depressing the intracellular generation of ROIs (Figure 6.3), failed to block the cytotoxic effects of TNF-α and IFN-γ on trophoblasts to any significant degree, although they were slightly effective ($p \leq 0.05$) in inhibiting cytotoxicity induced by TNF-α alone (Table 6.5).

Addition of sphingomyelinase (SMase) mimics the cytotoxic effects of TNF-α on trophoblasts. To investigate the possibility that ceramide mediates TNF-α-induced cytotoxicity, the intracellular levels of ceramide were manipulated pharmacologically by the exogenous addition of SMase. After 18 hours of culture, the viability of cells exposed to 1000 mU/ml of SMase was 24% of that of control cells cultured in medium alone, whereas exposure of cells to the vehicle had no adverse effect (Figure 6.4). The effect of SMase was reduced to control levels at 125 mU/ml.

Inhibitors of RNA and protein synthesis enhance cell death. In some cell lineages, such as thymocytes, the process of apoptosis follows an inductive mechanism in that expression of novel genes is required for cell death. In other models, such as in the cell line HL-60, the apoptotic pathway is constitutive but suppressed by inhibitory factors with half short-lives and thus is released upon inhibition of RNA and protein synthesis (Cohen, 1993). I therefore investigated which of the above two mechanisms is used by trophoblasts to undergo apoptosis. Figure 6.5 showed that column-purified trophoblasts in culture were remarkably sensitive to both act D and CHX: concentrations of these inhibitors that had no effects on placental fibroblasts killed virtually all the trophoblasts after 18 hours of incubation. At the highest concentration of CHX and act D that had no effect on trophoblast viability (300 ng/ml and 4 ng/ml, respectively), both inhibitors exacerbated cytokine-induced cell death, the extent of which was already apparent after 18 hours of culture. Table 6.6 showed that CHX did not significantly enhance the cytotoxic effects of TNF-α, but increased the degree of cell death by 30% ($p \leq 0.05$) when a combination of TNF-α and IFN-γ was present. On the other hand, act D enhanced both the effects of TNF-α and TNF-α plus IFN-γ by 10% and 80% respectively ($p \leq 0.005$). These results suggest that the mechanism of apoptosis in trophoblasts identifies more with the release pathway than the inductive pathway.

C. Summary of Results

1. The p55 TNF-R constitutes the major signal transducer for TNF-α-induced trophoblast death.

2. The addition of exogenous SMase, and not the generation of NO or ROIs, mimics TNF-α in terms of killing the trophoblasts, suggesting that the sphingomyelin pathway may transduce a cytotoxic signal for TNF-α in trophoblasts.
3. Trophoblasts are extremely sensitive to CHX and Act D; these inhibitors synergize with TNF-α and IFN-γ in enhancing cell death, suggesting that the apoptotic pathway in trophoblasts does not require the synthesis of new proteins. Rather the apoptotic machinery is constitutively suppressed by factors with short half-lives.

Table 6.1. Effects of the anti-TNF receptor antibodies htr-9 and utr-1 on TNF-α-induced cytotoxicity in cultured trophoblasts.

Treatments	MTT Readout (O.D.)		
	medium alone	+TNF	+ TNF + IFN
IgG1	0.400 ± 0.007^{a}	0.331 ± 0.019^{a}	0.282 ± 0.006^{a}
htr-9	0.327 ± 0.016^{b}	0.306 ± 0.012^{a}	0.242 ± 0.005^{a}
utr-1	0.384 ± 0.013^{a}	0.306 ± 0.012^{a}	0.262 ± 0.011^{a}
htr-9 + utr-1	0.328 ± 0.005^{b}	0.305 ± 0.009^{a}	0.252 ± 0.027^{a}

Column-purified trophoblasts were plated at $1x10^{5}$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, antibodies at 2 μg/ml were incubated with trophoblasts for 1-2 hours before the addition of TNF-α (10 ng/ml) or TNF-α plus IFN-γ (100 U/ml). Viability was assayed by MTT after 4 days in culture. Values reflect the mean ± S.D. performed in triplicate in one representative experiment out of three performed.

Means within a column with no common superscripts differ significantly ($p \leq 0.05$; Tukey's test).

Table 6.2. Effects of arginine on the cytotoxicity of trophoblasts mediated by TNF-α alone or in combination with IFN-γ.

Arginine concentration (μg/ml)	MTT Readout (O.D.) medium alone	+TNF	+TNF+IFN
0	0.323 ± 0.019[a]	0.250 ± 0.005 (77%)[a]	0.140 ± 0.007 (43%)[a]
20	0.345 ± 0.009[a]	0.252 ± 0.005 (73%)[a]	0.145 ± 0.004 (42%)[a]
200	0.364 ± 0.004[a]	0.271 ± 0.002 (74%)[a]	0.150 ± 0.010 (41%)[a]
2000	0.191 ± 0.040[b]	0.099 ± 0.032 (52%)[b]	0.079 ± 0.011 (41%)[b]

Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS, wells were washed three times with warm RPMI, and RPMI plus 10% dialyzed FCS reconstituted with increasing amounts of L-arginine prepared with or without TNF-α (10 ng/ml) or TNF-α plus IFN-γ (100 U/ml) was added to the cells. Viability was determined by MTT after 4 days in culture. Values represent the mean of triplicates ± S.D. Percentages were calculated relative to medium alone controls.

Means within a column with no common superscripts differ significantly ($p \leq 0.05$; Tukey's test).

Table 6.3. Effects of NO inhibitors on the cytotoxicity of trophoblasts in the presence of TNF-α alone or in combination with IFN-γ.

Reagents	MTT Readout (O.D.)		
	medium alone	+TNF	+TNF +IFN
IMDM	0.331 ± 0.027[a]	0.275 ± 0.006 (83%)[a]	0.207 ± 0.012 (63%)[a]
NAME	0.357 ± 0.019[a]	0.296 ± 0.014 (83%)[a]	0.219 ± 0.024 (61%)[a]
NMMA	0.352 ± 0.012[a]	0.313 ± 0.005 (89%)[b]	0.230 ± 0.010 (65%)[a]

Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, trophoblasts were incubated with NAME (5 mM final concentration) and NMMA (1mM final concentration) prepared in IMDM / 10% FCS for 1-2 hours before the addition of TNF-α (10 ng/ml) or TNF-α plus IFN-γ (100 U/ml). MTT was performed after 4 days of culture. Values presented are the mean ± S.D of triplicate determinations in one representative experiment out of 4 performed. Percentages were calculated relative to medium alone controls.

Means within a column with no common superscripts differ significantly ($p \leq 0.05$; Tukey's test).

Table 6.4. Generation of ROIs by trophoblasts treated with TNF-α and IFN-γ.

Duration of cytokine treatment (hours)	Arbitrary Fluorescence Units		
	IMDM	+TNF	+TNF +IFN
3	724 ± 30	715 ± 19	729 ± 8
18	763 ± 24	706 ± 24	596 ± 48

Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, trophoblasts were incubated with TNF-α (10 ng/ml) and IFN-γ (100 U/ml) for 18 hours or for 3 hours after culturing overnight culture in IMDM/ 10% FCS. The medium was aspirated, wells were washed three times with PBS, dichlorofluorescin diacetate (DCF-DA) prepared in Hanks balanced salt solution (HBSS) at a final concentration of 1 μg/ml was added and the fluorescence quantitated after 60 minutes with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Values represent the mean ± S.D. performed in triplicates in one out of 5 experiments performed with similar results. Trophoblasts derived from the same placenta cultured in the presence of TNF-α alone (10 ng/ml) or in combination with IFN-γ (100 U/ml) resulted in 25% and 32% viability loss respectively after 4 days of culture.

Table 6.5. The effect of ROI inhibitors on the cytotoxicity of trophoblasts stimulated by TNF-α alone or in combination with IFN-γ.

Reagents	MTT readout (O.D.)		
	medium alone	+TNF	+TNF+IFN
medium	0.403 ± 0.008^{a}	0.280 ± 0.005 (69%)^{a}	0.241 ± 0.006 (60%)^{a}
ascorbic acid	0.421 ± 0.029^{a}	0.322 ± 0.006 (76%)^{b}	0.238 ± 0.003 (57%)^{a}
BHA	0.444 ± 0.013^{a}	0.314 ± 0.011 (71%)^{b}	0.247 ± 0.005 (56%)^{a}

Column-purified trophoblasts were plated at $1x10^{5}$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells,, ascorbic acid and BHA (both at 100 μM final concentration) were prepared in IMDM /10% FCS, and incubated with the trophoblasts for 1-2 hours before the addition of TNF-α (10 ng/ml) or TNF-α plus IFN-γ (100 U/ml). Cells were then cultured for 4 days before performing a MTT assay. Values represent the mean of triplicate wells ± S.D. in one out of 6 experiments performed with reproducible results. Percentages were calculated relative to medium alone controls.

Means within a column with no common superscripts differ significantly ($p \leq 0.05$; Tukey's test).

Table 6.6. Effects of CHX and act D on trophoblasts cultured for 18 hours in the presence of TNF-α alone or in combination with IFN-γ.

Treatments	MTT Readout (O.D.)		
	medium alone	+TNF	+TNF + IFN
medium alone	0.370 ± 0.014^{a}	0.339 ± 0.006 (92%)^{a}	0.311 ± 0.007 (84%)^{a}
CHX (300 ng/ml)	0.368 ± 0.011^{a}	0.326 ± 0.010 (89%)^{a}	0.255 ± 0.015 (69%)^{b}
act D (4 ng/ml)	0.370 ± 0.010^{a}	0.309 ± 0.005 (84%)^{b}	0.172 ± 0.036 (46%)^{c}

Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, trophoblasts were incubated with CHX and act D at the indicated concentrations for 4 hours before addition of TNF-α (10 ng/ml) or TNF-α plus IFN-γ (100 U/ml). MTT assay was performed after a further 18 hours of incubation. Values are the mean of quadruplicate wells ± S.D. in one representative experiment out of 3 performed. Percentages were calculated relative to medium alone controls.

Means within a column with no common superscripts differ significantly ($p \leq 0.05$; Tukey's test).

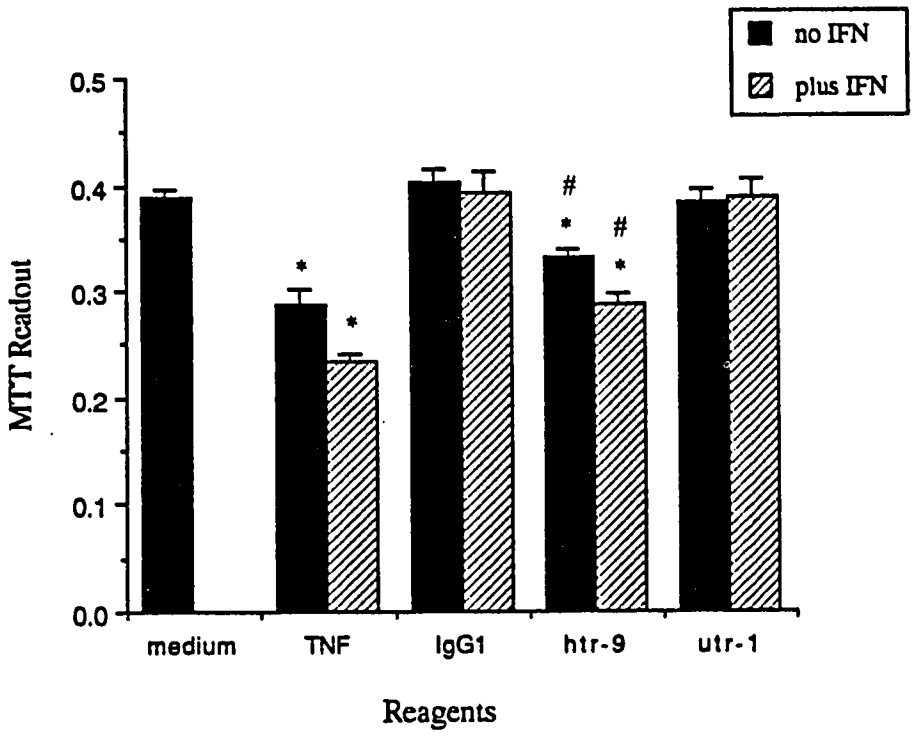


Figure 6.1. Effect of agonistic htr-9 and antagonistic utr-1 anti-TNF-R antibodies on the induction of cytotoxicity in trophoblasts. Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, trophoblasts were treated with TNF-α (10 ng/ml) or antibodies at 2 μg/ml, with or without of IFN-γ (100 U/ml). MTT reductive capacity was measured after 4 days of culture. Each bar represents the mean ± S.D. of triplicates in one out of 4 experiments performed with similar results. This experiment was repeated twice with similar results.

*$p \leq 0.05$, compared with their medium alone controls (Tukey's test).

#$p \leq 0.05$, compared with their respective TNF alone values (Tukey's test).

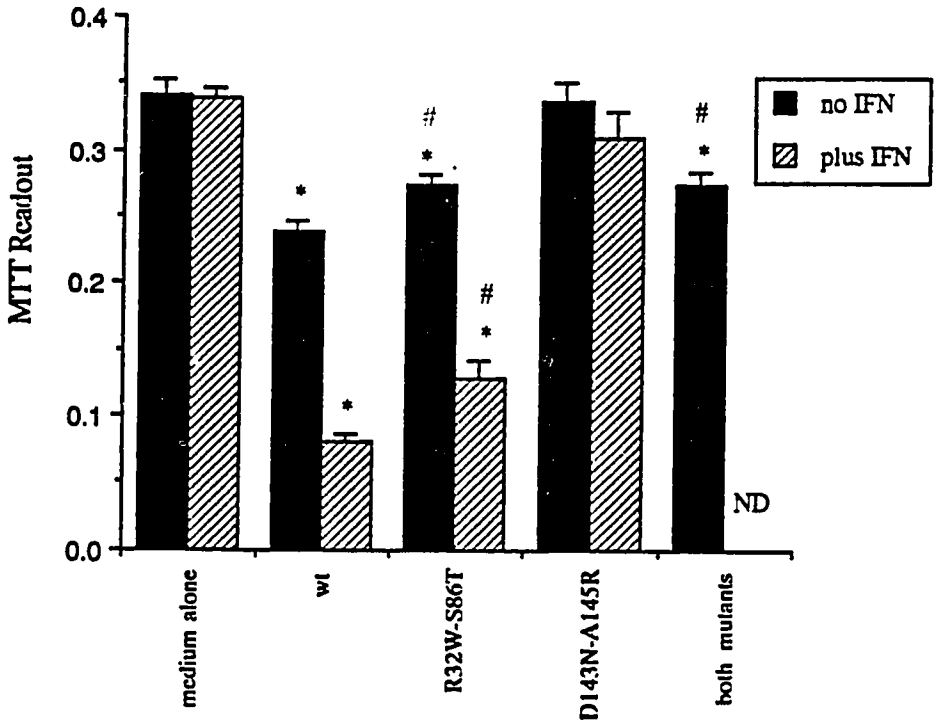


Figure 6.2. Cytotoxic activity of human wild-type TNF-α and TNF-α mutants in trophoblasts. Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, trophoblasts were incubated with either wild type (wt) TNF-α, TNF-α mutant with selective binding to p55 (R32W-S86T) or to p75 (D143N-A145R), or a combination of both mutants. All TNF-α concentrations were used at 50 ng/ml and in some wells, IFN-γ was added at a final concentration of 100 U/ml. Viability was determined after 4 days using MTT. Each bar represents the mean ± S.D. of triplicates in one out of 2 experiments performed.
ND= not done.
*$p \leq 0.05$, compared with their respective medium alone controls (Tukey's test).
#$p \leq 0.05$, compared with their respective wt TNF alone values (Tukey's test).

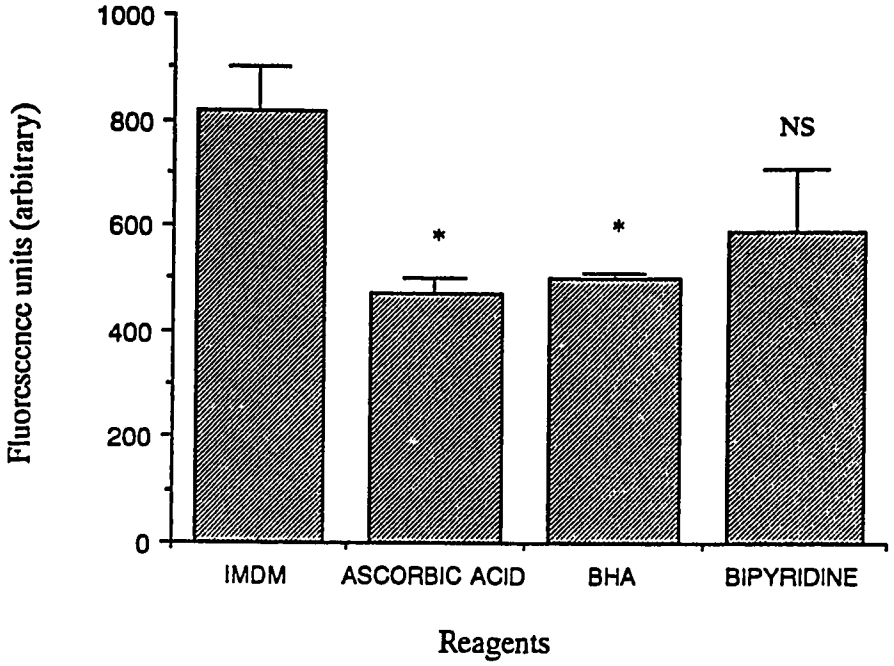


Figure 6.3. Inhibition of ROIs generation by ascorbic acid, BHA and bipyridine. Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, trophoblasts were then cultured for 18 hours. Ascorbic acid (100 μM), BHA (100 μM) and bipyridine (30 μM) were added during the final 3 hours of culture. Medium was aspirated and after extensive washing with PBS, DCF-DA prepared in HBSS was added and fluorescence quantitated at 60 minutes as described in Materials and Methods. Each bar represents the mean of triplicate wells ± S.D after subtraction of the control, which consisted of DCF-DA in HBSS alone. This experiment has been repeated 8 times; the one shown here is representative.

*$p \leq 0.05$ when compared to IMDM values

NS= non-significant as compared to IMDM values.

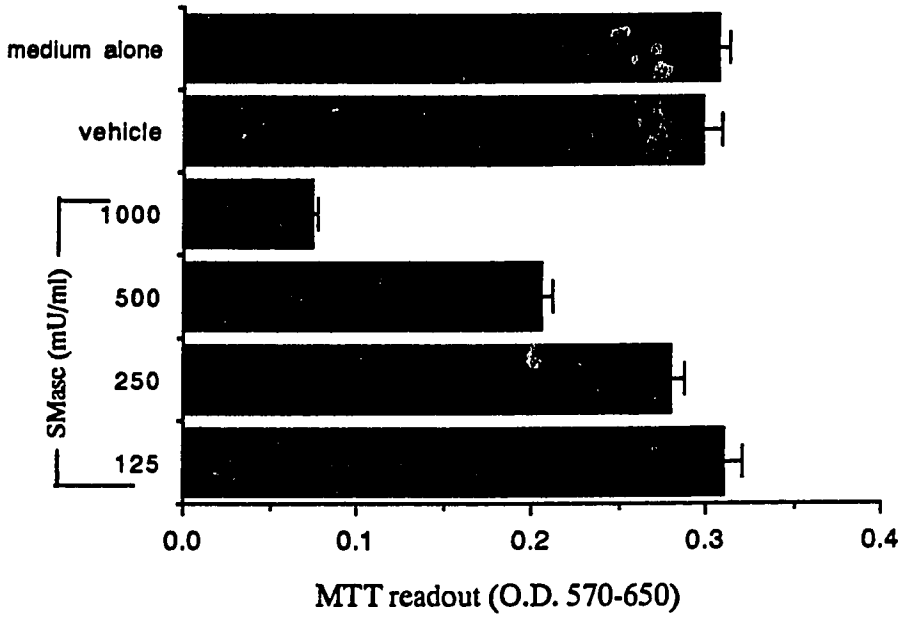


Figure 6.4. Concentration dependency of SMase in the induction of trophoblast cytotoxicity. Column-purified trophoblasts were plated at $1x10^5$ cells per well in 96-well tissue culture plates. After adherence in IMDM/ 10% FCS and washing away non-adherent cells, trophoblasts were cultured in IMDM/ 10% FCS (medium alone), in vehicle alone, and in various concentrations of SMase. Viability was determined by MTT after 18 hours of culture. Each bar represents the mean ± S.D of triplicate determinations in one out of 3 experiments performed with similar results.

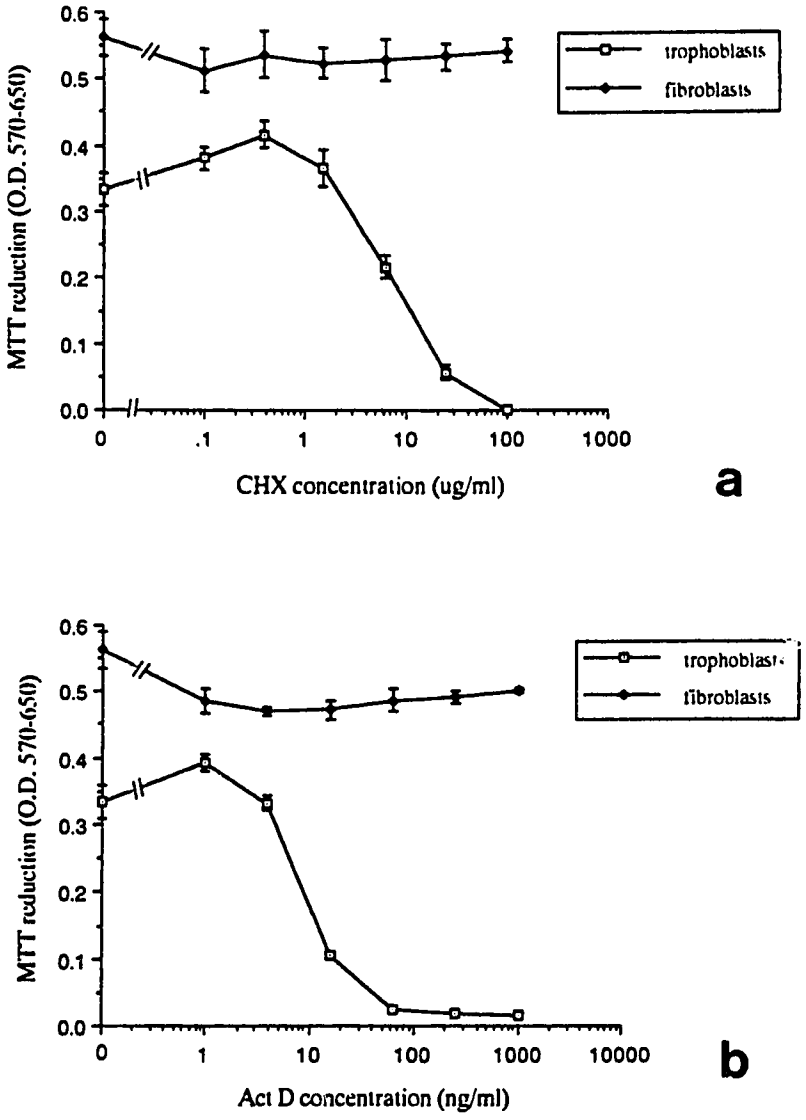


Figure 6.5. Viability of placental trophoblasts and fibroblasts as a function of the concentration of (a) CHX and (b) act D. Placental trophoblasts were seeded at 1x 10^5 cells and fibroblasts at 2 x 10^4 cells per 100 µl and allowed to adhere for 4 hours in IMDM/ 10% FCS. After washing away non-adherent cells, cells were incubated with CHX and act D. MTT reduction was assessed after 18 hours in culture. This experiment was repeated twice with similar results. Each point represents the mean of triplicate wells ± S.D.

CHAPTER VII

ANTAGONISM OF TNF-α-INDUCED CYTOTOXICITY BY EGF AND GLUCOCORTICOIDS

A. Introduction

The observation that TNF-α induces the apoptosis of villous CTs implies that the expression of this cytokine may require stringent control at the maternal-fetal interface. Otherwise untimely depletion of these stem cells that form the ST may have severe consequences in the integrity of the placental barrier. One mechanism of control can be at the level of transcription or translation of the TNF-α gene. Evidence from *in situ* hybridization studies has shown gestation-related fluctuations in the expression of TNF-α mRNA and protein (Chen et al., 1991). Another mechanism of control could be via suppression of TNF-α activities by other factors synthesized at the maternal-fetal interface.

One class of compounds known to inhibit the cytotoxic effects of TNF-α are the glucocorticoid steroids. It has been shown that the administration of dexamethasone (dex) confers protection to TNF-α-induced septic shock and subsequent mortality in adrenalectomized mice (Bertini, Bianchi and Ghezzi, 1988). Since glucocorticoids bind to cytoplasmic receptors to initiate their protective effects, the glucocorticoid receptor antagonist RU 38486 sensitizes rodents to low doses of TNF-α (Brouckaert, Everaedt and Fiers, 1992). In addition, dex also suppresses the cytotoxic activity of TNF-α on fibroblasts and adipocytes cultured *in vitro* (Suffys et al., 1987; Reid, Torti and Ringold, 1989).

In addition to steroids, studies in murine pregnancy indicate other cytokines may be able to counteract the harmful effects of TNF-α and IFN-γ. Thus, mice undergoing high rates of spontaneous resorptions, likely to be mediated by enhanced levels of TNF-α and IFN-γ expression (Tangri and Ragupathy, 1993), regained normal litter sizes and placental and fetal weights upon *in vivo* administration of GM-CSF and IL-3 (Chaouat et al., 1990). Another cytokine which may show beneficial effects is EGF. It has been shown that EGF induces the formation of syncytium (Morrish et al., 1987) and stimulates increase of hCG and hPL, two hormones vital for fetal growth and survival (Maruo et al., 1987; Morrish et al., 1987; Barnea et al., 1990). Low levels of EGF also appear to be associated with delivery of IUGR babies (Hofmann et al., 1988).

Since the placenta is rich in its synthesis of cytokines, glucocorticoids and other types of steroid hormones (Pasqualini and Kincl, 1985), and they play a crucial role in the maintenance and regulation of placental functions, the objective of this chapter is to investigate whether the cytotoxic action of TNF-α on primary cultured trophoblasts can be inhibited by dex, as an example of a glucocorticoid, and to initiate a preliminary search for cytokines that can suppress the cytotoxic effects of TNF-α *in vitro.*

B. Results

Dexamethasone (dex) is effective in inhibiting TNF-α-induced cytotoxicity. In several cell systems, glucocorticoids are known to suppress the cytotoxicity induced by TNF-α (Reid, Torti and Ringold, 1989). In trophoblasts, I found that dex was also effective in reversing the cytotoxic effects of TNF-α in a dose-dependent manner, with half maximal inhibitory activity between 5-10 nM (Figure 7.1). This concentration is in accord with that required to suppress TNF-α-induced cytotoxicity in other cell systems (Reid, Torti and Ringold, 1989). When dex was added in the presence of both TNF-α and IFN-γ, it also promoted the viability of the cultures to 90% of that seen in control cultures.

EGF-treated cultures demonstrate increased resistance to cytokine-induced cytotoxicity. The cytokines GM-CSF, CSF-1 and EGF have been previously reported to affect pregnancy in a beneficial maner (see references in introductory section of this chapter). They were therefore examined for their ability to inhibit TNF-induced trophoblast death and EGF was found to be most effective in counteracting the cytotoxic effects of TNF-α and IFN-γ (Figure 7.2). To further assess the effects of EGF on cultured trophoblasts in the presence of TNF-α and IFN-γ, increasing concentrations of EGF (0-10 ng/ml) were added to the cultures treated with TNF-α and IFN-γ. Table 7.1 showed that EGF at 10 ng/ml EGF completely blocked the cytotoxic effects of TNF-α and increased the survival rate of cultures treated with TNF plus IFN-γ by 44%. Thus EGF present

simultaneously with TNF-α or TNF-α plus IFN-γ can protect cells from the TNF-induced apoptosis.

Dexamethasone and EGF prevent cell death by inhibiting apoptosis. In Chapter 5, I reported that trophoblasts died via apoptosis upon exposure to TNF-α alone or in combination with IFN-γ. I therefore investigated whether dex and EGF blocked TNF-induced cytotoxicity by inhibiting apoptosis. Cells were treated with either dex or EGF, then cultured in the presence of TNF-α alone or with IFN-γ for 18 hours and the number of apoptotic cells scored using the TUNEL technique. Figure 7.3 showed that in cultures treated with dex or EGF, the percentage of apoptotic nuclei decreased by 50% as compared to control cultures. The low numbers of apoptotic nuclei observed in cultures without the addition of TNF-α and IFN-γ addition could reflect spontaneous cell death occuring during the time of culture or they could result from DNA nicking introduced during the experimental procedure.

C. Summary of Results

1. Glucocorticoids (of which dexamethasone is an example) and EGF, factors normally present in normal placenta, promote cytotrophoblast survival in the presence of TNF-α alone or in combination with IFN-γ.
2. Reduction in apoptotic nuclei constitutes part of the counteracting effects of dex and EGF against TNF-α induced cytotoxicity.

Table 7.1. Effect of EGF on TNF-α-induced cytotoxicity of cultured trophoblasts.

EGF concentration (ng/ml)	MTT readout (O.D.) IMDM	+TNF	+TNF + IFN
0.0	0.315 ± 0.016^{a}	0.274 ± 0.004 (87%)^{c}	0.206 ± 0.010 (65%)^{c}
0.1	0.323 ± 0.007^{a}	0.294 ± 0.006 (91%)^{b}	0.229 ± 0.013 (71%)^{b}
1.0	0.319 ± 0.012^{a}	0.296 ± 0.006 (93%)^{b}	0.242 ± 0.013 (76%)^{b}
10.0	0.329 ± 0.008^{a}	0.329 ± 0.003 (100%)^{a}	0.308 ± 0.007 (94%)^{a}

Column-purified trophoblasts were plated at $1x10^{5}$ cells in 96 well tissue culture plates. After a 4 hour adherence step and washing away non-adherent cells, trophoblasts were incubated with EGF at increasing concentrations for 1-2 hours before the addition of TNF-α alone (10 ng/ml) or in combination with IFN-γ (100 U/ml). Cell viability was determined 4 days later by MTT. Values represent the mean of triplicates ± S.D. Percentages were calculated relative to medium alone controls.

Means within a column with no common superscripts differ significantly ($p \leq 0.05$, Tukey's test).

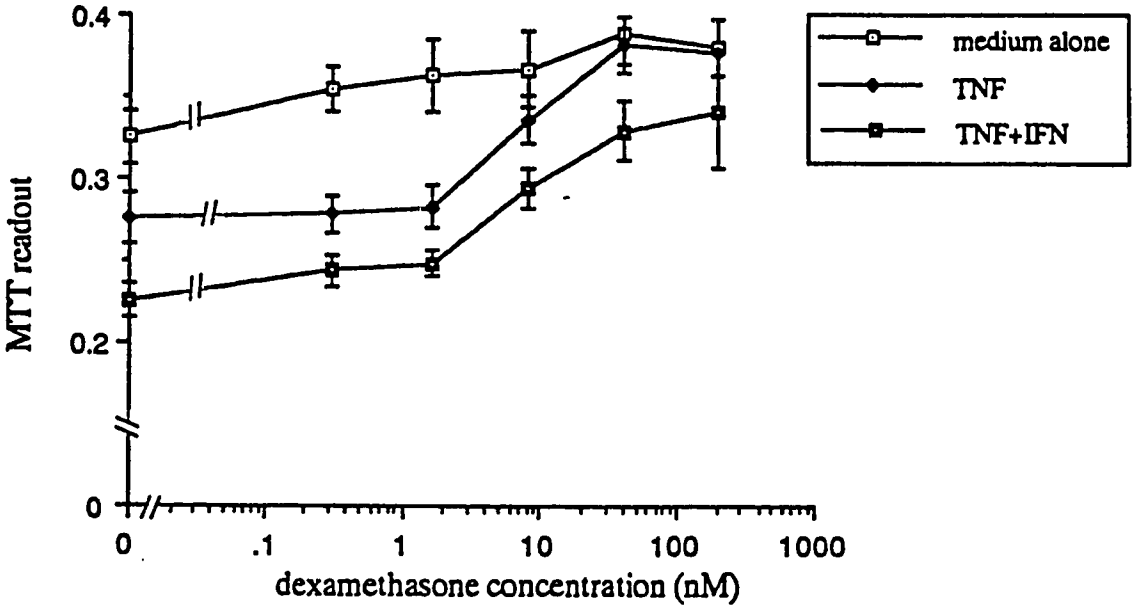


Figure 7.1. Effect of dexamethasone on TNF-α/ IFN-γ-induced cytotoxicity of cultured trophoblasts. Column-purified trophoblasts were plated at $1x10^5$ cells in 96 well tissue culture plates. After a 4 hour adherence step and washing away non-adherent cells, trophoblasts were incubated with four-fold dilutions of dex for 5 hours before the addition of TNF-α (10 ng/ml) or TNF-α plus IFN-γ (100 U/ml), and cell viability was determined by MTT after 4 days of culture. Each point represents the mean of triplicate wells ± S.D. in one out of 4 experiments performed with similar results.

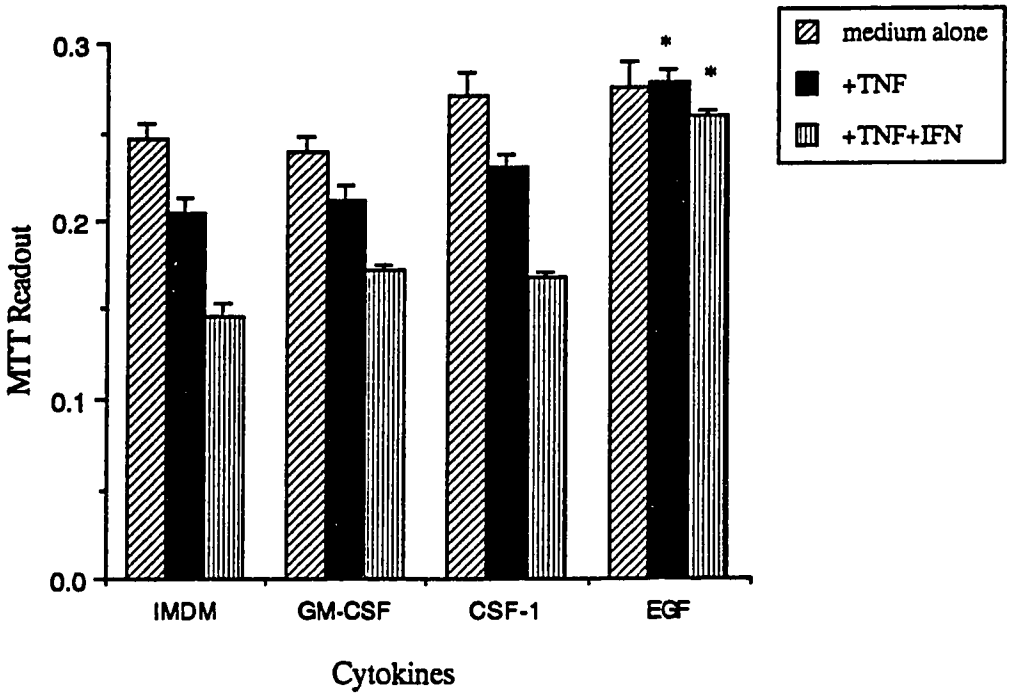


Figure 7.2. Effects of GM-CSF, CSF-1 and EGF on TNF-α/ IFN-γ-induced cytotoxicity of trophoblasts. Column-purified trophoblasts were plated at $1x10^5$ cells in 96 well tissue culture plates. After a 4 hour adherence step and washing away non-adherent cells, trophoblasts were incubated with GM-CSF (10 ng/ml), CSF-1 (10,000 U/ml) and EGF (10 ng/ml) for 1-2 hours before the addition of TNF alone (10 ng/ml) or in combination with IFN-γ (100 U/ml). Viability was determined after 4 days by MTT. Bars represent the mean of triplicate wells ± S.D.

*$p \leq 0.005$, compared with their respective values in IMDM (student t-test).

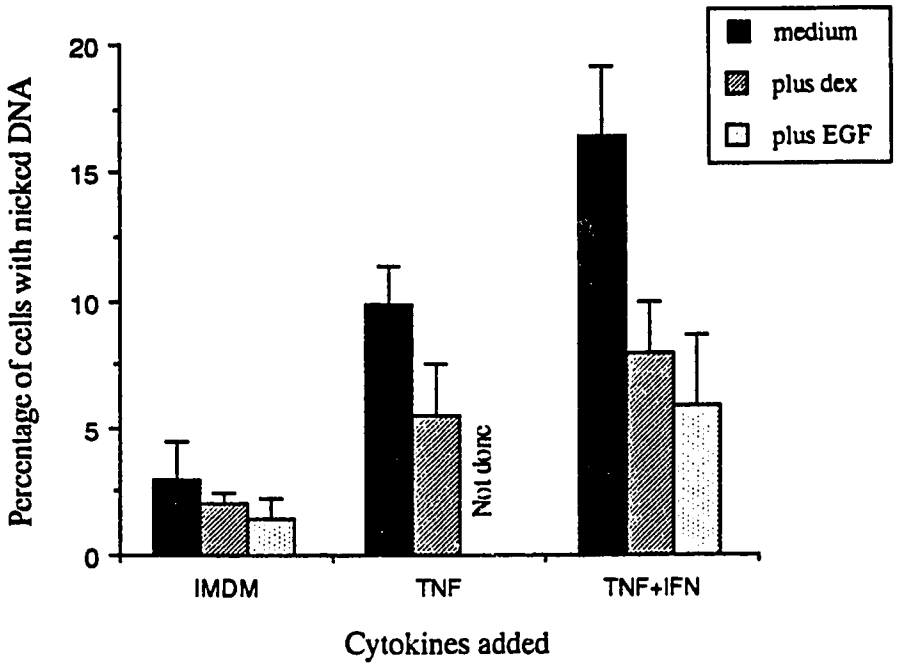


Figure 7.3. Reduction in the numbers of apoptotic nuclei in cultures treated with dexamethasone and EGF. Column-purified trophoblasts were plated at $1x10^5$ cells in 96 well tissue culture plates. After a 4 hour adherence step and washing away non-adherent cells, trophoblasts were incubated with dex (200 nM) for 6 hours or EGF (10 ng/ml) for 1-2 hours before the addition of TNF-α (10 ng/ml) and TNF-α plus IFN-γ (100 U/ml). Cultures were then incubated for a further 18 hours before performing TUNEL. A total of 1200-1600 cells from 8 different fields in 2 separate experiments were counted.

CHAPTER VIII
DISCUSSION

A. Introduction

The process of human placentation, which encompasses cellular invasion, cell-matrix interactions, tissue remodeling, cellular proliferation, differentiation and functional specialization, is a tightly orchestrated event demanding the co-ordinated interactions of multiple cells of both maternal and fetal origins. Lymphohematopoietic cytokines, via their versatile and redundant activities, play a major role in facilitating the intercellular communication that underlies such processes. In murine pregnancy, a concept has emerged that cytokines can be classified into two camps: those which are beneficial to placental growth and fetal survival, and those which are deleterious to the well-being of the feto-placental unit (Wegmann, 1990). Recent experimental evidence indicates that a successful pregnancy is tilted towards the production of Th2 type cytokines, whereas Th1 cytokines are harmful to fetal survival, resulting in fetal resorptions (Hui et al., 1993; Wegmann et al., 1993).

It awaits to be verified whether such a distinctive pattern exists in human reproduction. Nonetheless, immunohistochemical staining and *in situ* hybridization studies have localized the production of cytokines and their corresponding receptors to both immune and non-immune cellular components of the human placenta, suggesting the presence of a localized cytokine network (Mitchell, Trautman and Dudley, 1993). Despite a growing literature confirming the expression of cytokines, studies directed at the effects of cytokines on the proliferation, differentiation and function of a

particular placental cell type are relatively few and are only now emerging in the literature. Until recently, the emphasis of most studies in humans has been on cytokine-endocrine interactions, such as IL-1 influence on hCG and hPL secretion (Berkowitz et al., 1988; Yagel et al., 1988; Silen et al., 1989; Hill, 1992) or cytokine modulation of surface receptors such as the effects of IFN-γ on HLA expression (Hunt and Orr, 1992).

Previous work has demonstrated the effects of TNF-α on murine and rat trophoblasts as well as human choriocarcinoma cell lines, but its activity on primary human placental trophoblasts remains un-investigated. I therefore initiated an *in vitro* study to examine the effects of TNF-α on a purified population of human trophoblasts obtained from term placenta. My results showed that TNF-α induced cytotoxicity in cultured trophoblasts and that IFN-γ, as well as inhibitors of RNA and protein synthesis, synergized with TNF-α to enhance the killing. In return, trophoblasts responded by undergoing apoptosis. The signaling pathway appeared to involve predominantly the p55 TNF-Rs. SMase mimicked the cytotoxic effects of TNF-α, suggesting the possibility of a ceramide-dependent pathway. In addition, dexamethasone and EGF suppressed the cytotoxicity of TNF-α on trophoblasts.

B. Development of an *in vitro* culture model to study TNF-α effects on placental trophoblasts

In the introductory chapter, it was mentioned that trophoblasts were present in the placental villi intermingled with other cells including fibroblasts, macrophages and endothelial cells. When crude, unpurified placental cells were placed in culture, fibroblasts tend to overgrow

trophoblast cultures (Yeger et al., 1989). Thus in order to study the effects of TNF-α on trophoblasts, my first step was to develop a defined *in vitro* culture model using pure trophoblast cells. Since existing purification protocols were unsatisfactory because of low yields and compromised cell viability, I proceeded to develop a new method for obtaining trophoblasts from term placenta with improved yields and viability.

The purification procedure described in chapter IV utilizing glass bead columns is efficient (total processing time takes 6 hours), highly reproducible and gives both a good yield of mononucleated trophoblasts and effectively decreases the numbers of non-trophoblastic cells from preparations derived from term villi (Table 4.1). The Percoll gradient step that is often used with immuno-magnetic bead methods (Douglas and King, 1989; Bischof et al., 1991) has been replaced with a simple red cell lysis step, thereby eliminating cellular aggregates often associated with Percoll usage (Karl, Alpy and Fisher, 1992) as well as increasing the yield before further purification from the reported 30 to 60 million cells to 140 million cells per 30 grams of tissue (Table 4.1). The observation that cells expressing Leu M5 or HLA class I are removed with those expressing CD9 implies that these antigens co-express with CD9 on mesenchymal cells. Placing the purified cells into culture further reduces the frequency of CD9 positive cells (<0.05%), numbers which are in agreement with the very low level of vimentin positive cells (0.3%) found in the cultures (Table 4.2). That these trophoblasts are isolated and purified from chorionic villous tissue, and that they express cytokeratin (Figure 4.3) and produce hCG (Figure 4.5) but lack HLA class I, indicate the purified population to be villous rather than extravillous trophoblasts, such as placental bed giant cells (Loke and Butterworth, 1987). The column purified trophoblasts are >99% viable directly after column purification, and upon

cryopreservation and thawing still maintain an all-over plating efficiency of around 80%. In cultures of up to 2 weeks, the adhering trophoblasts survive, retain full metabolic capacity and function, and the very low levels of contaminating mesenchymal cells do not increase (Table 4.3).

When placed in serum-supplemented medium, the isolated cells formed a monolayer reminiscent of a syncytial sheet. However, anti-desmoplakin staining revealed that the majority of cells remained as unfused, mononucleated trophoblasts (Figure 4.6). This was also supported by microinjection of FITC-dextran into cellular units, where the fluorescent dye remained localized and did not diffuse into adjacent cells through gap junctions (Yui et al., 1994). The observation that column-purified trophoblasts remained mononucleated throughout a 1-week culture period indicates that either trophoblasts are somewhat heterogeneous in their intrinsic capacity to fuse, or that optimal culture conditions, such as the use of keratinocyte culture medium (Douglas and King, 1990) and/ or a combination of extracellular matrix proteins and supplemental growth factors (Kao et al., 1988) are required to achieve uniform syncytialization *in vitro*. Indeed when EGF was added to the culture medium, the proportion of syncytial structures markedly increased (Figures 4.6 and 4.7). This observation is also in agreement with the report that EGF induced the differentiation of term, but not first trimester CTs into ST under serum-free conditions (Morrish et al., 1987; Barnea et al., 1990). However, one difference between my results with those of Morrish et al. (1987) concerns the degree of syncytialization. Whereas Morrish et al. (1987) observed complete syncytialization by day 7 of culture, in my system, bi- or multi-nucleated syncytial units consisted of only 50% of the total cells in culture. This discrepancy can be due to the presence of serum in the medium, the difference in cell purification protocols, and most

likely, the density of cells seeded. As judging from the photomicrographs, Morrish et al. (1987) had fewer cells in their cultures. Preliminary experiments in this laboratory found that low cell densities favored the formation of syncytium. However at present, it is unclear as to whether the accumulation of inhibitory factors of trophoblast differentiation, such as TGF-β1 (Morrish, Bhardwaj and Paras, 1991), and/ or spatial limitation cause reduced syncytialization at high seeding density. Clearly, the parameters governing the formation of a syncytial monolayer from mononucleated trophoblasts deserve further investigation.

It is generally accepted that hCG production is an exclusive product of the ST, and that hCG production *in vitro* is a consequence of syncytium formation (Kliman et al., 1986; Morrish et al., 1987). Here I showed that trophoblasts cultured in serum-supplemented medium for a week stained positively for hCG and secreted hCG into the medium during the first 4 days of culture without the apparent formation of multinucleated syncytium (Figure 4.5). These results supported the hypothesis that trophoblasts of the chorionic villi consisted of mono- and multi-nucleated cells in a continuum of differentiated stages and that the progression of mononucleated CTs to multinucleated ST is only one part of the trophoblast differentiation program and thus can be dissociated from biochemical differentiation (Kao et al., 1988). In this aspect, the mononucleated trophoblasts after 7 and 14 days of culture could be biochemically, if not phenotypically, distinct from those at day 0 of culture.

Interestingly there appears to be a significant loss of DNA during the first week of culture without a corresponding loss in metabolic capacity (MTT reductive cleavage) and protein content (Table 4.3). This phenomenon could reflect concomitant processes of cell loss and cellular differentiation to

a larger, more activated cell type. The maturation of blood monocytes to tissue macrophages (Beelen et al., 1989) provides a good model for such differentiation, as indeed are mononuclear phagocytes good models for trophoblast in general (Guilbert, Robertson and Wegmann, 1993). Alternatively, the loss of DNA without loss of other cellular constituents may be part of the differentiation process to form syncytium. Others have reported that inhibition of DNA synthesis occurs after the first 24 hours in culture (Ohlsson, 1989). Whether this is a phenomenon pertaining to all cells in culture or restricted to isolated groups of cells demands further investigation, and the antibody Ki-67 which is directed against a nuclear antigen associated with proliferating cells may prove useful in clarification of this matter (Gerdes et al., 1983). On the other hand, the low thymidine uptake values could reflect a process of DNA repair. The observations that the DNA content of the cultures does not increase and that DNA synthesis does not occur is also in accord with previous observations that trophoblasts from term placenta do not proliferate and that syncytium forms through fusion rather than by endomitosis (Kliman et al., 1986; Douglas and King, 1990).

In conclusion, I have developed and characterized an *in vitro* model of culturing trophoblasts to be used subsequently in examining the effects of TNF-α on these cells. The improved yields and high degree of purity of the trophoblast preparations described here may offer a distinct advantage to a number of other studies. One example involves understanding the mechanisms leading to syncytialization of the villous trophoblast. Contaminating stromal cells, particularly fibroblasts and macrophages, produce extracellular matrix proteins and cytokines (Hunt, 1989; Autio-Harmainen et al., 1991), and may therefore obscure the assessment of direct effects of these factors on trophoblast differentiation. This same caveat is

valid for determining the effects of other biological response modifiers on trophoblast hormone production. Maximal elimination of non-trophoblastic cells is also important in studies of transport of macromolecules across trophoblast monolayers cultured on biological or synthetic support matrices. Since it has been reported that trophoblasts do not fuse with other cell types (Kliman et al., 1989), the variable presence of contaminating cells may explain in part the leak component observed in some of these studies (Bullen et al., 1990). On the other hand, the presence of macrophages or other non-trophoblastic cells bearing high affinity Fc receptors may hamper *in vitro* assessment of the extent and kinetics of Ig transfer across the trophoblast syncytium (Sooranna and Contractor, 1991). Finally, stromal cells are targets of specific pathogens (e.g. cytomegalovirus infects fibroblasts, HIV infects macrophages) and thus even low level contamination with these cells may confound the analysis of the precise role of the trophoblast in the transplacental passage of infectious agents, as illustrated by the current controversy regarding the permissiveness of trophoblast preparations to be infected *in vitro* with cell free HIV (Douglas and King, 1992).

C. TNF-α induces apoptosis in primary human placental cytotrophoblasts.

With the improvements in trophoblast purification by employing the negative selection of CD9 positive mesenchymal cells, I then proceeded to examine the effects of TNF-α on a population of CTs with greater than 99% purity in culture. In chapter V, I demonstrated that TNF-α alone and its combination with IFN-γ were detrimental to cytotrophoblast survival, the latter resulting in a 60% loss in the DNA content within 4 days of culture

(Table 5.1). The observation that this cytokine-induced damage is via apoptosis (Figures 5.5-5.7), a form of cell death that participates in tissue homeostasis, suggests that this process may serve a normal function. The observation that EGF-induced syncytium also undergoes apoptosis upon treatment with TNF-α and IFN-γ (Figure 5.8) suggests that these cytokines when originating from the maternal side may also directly affect the regulated turnover of this tissue or damage the syncytial barrier if levels are unregulated.

TNF-α kills a variety of tumor cells both *in vitro* and *in vivo,* but normal cells are generally resistant to the cytotoxic effects of this cytokine (Sugarman et al., 1985). Notable exceptions to this rule are the reported cytotoxicity of TNF-α towards bovine oligodendrocytes (Selmaj et al., 1991), human pancreatic islet cells (Rabinovitch et al., 1990) and, as demonstrated in this study, primary human cytotrophoblasts. The synergistic effects of IFN-γ on TNF-α mediated cytotoxicity reported in this study were also observed with islet cells (Rabinovitch et al., 1990). The cytotoxic effect of TNF-α on cytotrophoblasts was concentration-dependent with half-maximal effects at 3 ng/ml when used alone and at 1 ng/ml when used in combination with IFN-γ (Figure 5.2). These concentrations (3 ng/ml and 1 ng/ml) are equivalent to 0.2 nM and 0.06 nM respectively, which are within the reported K_d for the binding of TNF-α to its high affinity receptors, suggesting that engagement of high affinity receptors at low occupancy are sufficient in triggering cytotoxicity. Although the physiological levels of TNF-α and/ or IFN-γ at the feto-maternal interface during normal gestation are at present unknown, recent studies have demonstrated that TNF-α is undetectable in the amniotic fluid of normal pregnancies but can rise to 20 ng/ml in pregnancies complicated by preterm labor and infections (Casey et al., 1989). Consistent

with these results is the observation that explants of human decidua or fetal chorionic villous tissue produce very low amounts of TNF-α that can be enhanced in the presence of bacterial LPS (Vince et al., 1992). Thus, it appears that sufficient TNF-α levels in pathological situations could induce the death of trophoblasts.

IFN-γ enhances many of the biological activities of TNF-α including fibroblast proliferation (Hunt, Pace and Atherton, 1990), tumor cytotoxicity (Sugarman et al., 1985), upregulation of major histocompatability complex (MHC) class I expression (Hunt, Pace and Atherton, 1990), and as shown here, the cytotoxicity of trophoblasts. The mechanism of such synergism is unknown. Although it has been proposed that an increase in TNF-Rs induced by IFN-γ can be partly responsible for these effects (Aggarwal, Eessalu and Hass, 1985; Ruggiero et al., 1986), later studies demonstrated no correlation between susceptibility and the number of TNF-Rs or their affinity for TNF-α (Tsujimoto, Yip and Vilcek, 1985). In addition, the effects of TNF-α can be mediated by occupation of less than 15% of the total number of receptors (Thoma et al., 1990). In this regard, the synergy contributed by IFN-γ may involve sharing of post-receptor signaling components with those triggered by TNF-α, as both cytokines have been shown to activate sphingomyelin hydrolysis (Kim et al., 1991).

A consistent observation in these studies was that 60% of the trophoblasts were resistant to cytotoxicity mediated by TNF-α alone (Table 5.1). Neither increasing the concentration of TNF-α nor the time of exposure could overcome this lack of sensitivity (Figure 5.2). It is possible that while the analyzed population of cytotrophoblasts was homogeneous by all available morphological and immunohistochemical criteria, subtle differences related to the state of differentiation contributed to this differential sensitivity

to TNF-α. Alternatively, the heterogeneous expression of TNF-Rs by villous trophoblasts *in vivo* (Yelavarthi and Hunt, 1993) may be reflected in a population of resistant trophoblasts *in vitro*.

Although TNF-α induces both apoptotic and necrotic forms of cell death (Laster, Wood and Gooding, 1988), the distinction between these two processes is relevant to the understanding of the role of TNF-α in placental development. Unlike necrosis, which is death generally caused by external injury, apoptosis has been linked to the normal process of tissue homeostasis (Cohen, 1993). Therefore, the observation that TNF-α induces apoptosis in trophoblastic cells may shed light on the role of this cytokine in normal placental development. Ultrastructural examination of aggregates of syncytial nuclei in the term placenta often shows features characteristic of apoptosis, with chromatin condensation and severe pyknosis, which the authors attributed to cell senescence (Martin and Spicer, 1973). In addition, the increased expression of TNF-α mRNA and protein in chorionic tissues bears a direct correlation with the diminution of cytotrophoblastic layer underneath the syncytium as gestation progresses towards term (Chen et al., 1991). The molecular mechanisms that drive this process are largely unknown but the temporal correlation between increasing levels of TNF-α expression in the villous stroma and the diminishing pool of trophoblasts suggests that TNF-α may play a role in villous remodeling.

D. TNF-α mediates cytotoxicity in cultured cytotrophoblasts via p55 TNF receptors.

What is the mechanism by which TNF-α exerts its cytotoxic effects on trophoblasts? Delineating the components utilized by TNF-α to transduce the

cytotoxic signal may provide some insight into its inhibition by protective steroids and cytokines (see Chapter VII) and into therapeutic interventions to prevent aberrant TNF-α activity. In Chapter VI, I demonstrated that the p55 TNF-R played the major role in the transduction of cytotoxic signal initiated by TNF-α binding to trophoblasts by using a combination of receptor-specific monoclonal antibodies (Figure 6.1) and TNF-α mutants (Figure 6.2). A component of the sphingomyelin pathway, namely SMase, reproduced the cytotoxic effects of TNF-α and IFN-γ (Figure 6.4). Furthermore, trophoblast survival was extremely sensitive to transcriptional and translational inhibitors (Figure 6.5) and these inhibitors enhanced the cytotoxic effects of TNF-α and IFN-γ (Table 6.6).

Previous work with htr-9 (anti-p55) and utr-1 (anti-p75) have demonstrated their high-affinity binding to TNF-Rs and their abilities to mimic or block the biological effects of TNF-α in several systems. Thus, htr-9 which is agonistic, mediates TNF-α activities such as cytotoxicity in U937 cells, proliferation of fibroblasts, IL-6 production and adhesiveness in human umbilical cord endothelial cells (Espevik et al., 1990; Shalaby et al., 1990). On the other hand, utr-1 antagonizes some of these activities. Our observation that htr-9 alone reproduced TNF-mediated cytotoxicity in trophoblasts (Figure 6.1), whereas utr-1 failed to block TNF-induced cytotoxicity (Table 6.1), suggests that the cytotoxic signal in trophoblasts is transduced largely through p55 TNF-Rs. This finding is in accordance with the notion that p55 mediates most of the biological activities of TNF-α, whereas p75 has a limited function (Loetscher et al., 1993). A possible molecular explanation for this observation could be that p55, and not p75, is coupled to signal transduction cascades, particularly to those which employ lipid secondary messengers, such as phospholipase C (PLC), phospholipase A_2 (PLA_2) and

SMase (Wiegmann et al., 1992; Yanaga and Watson, 1992). Among these pathways it has been shown that the hydrolysis of sphingomyelin by SMase to yield ceramide induces apoptotic cell death, whereas the activation of PLA_2 or PLC fails to elicit cell shrinkage, chromatin compaction and DNA fragmentation into nucleosomal size fragments (Jarvis et al., 1994). Accordingly, I also showed that the exogenous addition of SMase led to rapid cell death within 18 hours (Figure 6.4), suggesting that trophoblasts may contain a neutral SMase which is activated upon TNF-α binding to p55. This finding, however, needs to be reinforced by measurement of a decrease in sphingomyelin content and a concomitant increase in ceramide after TNF-α stimulation of the trophoblasts. In addition, blocking TNF-α induced cytotoxicity in trophoblasts by a specific inhibitor of SMase would also contribute to a further piece of evidence that sphingomyelin hydrolysis is a signaling pathway for TNF-α-induced cytotoxicity in trophoblasts.

The inability of the agonistic htr-9 antibodies, even at high concentrations, to elicit cytotoxicity to the same extent as 10 ng/ml TNF-α (Figure 6.1) is also observed in other studies (Espevik et al., 1990; Greenblatt and Elias, 1992). This could be related to the valency of the antibodies in terms of proper engagement of the receptors as htr-9 belongs to the IgG-1 rather than IgM isotype. On the other hand, the inability of utr-1 to block TNF-α cytotoxicity cannot lead us to rule out the involvement of p75 in mediating cytotoxicity. First, it is possible that utr-1 recognizes an epitope outside the one mediating cytotoxicity, and therefore testing other antibodies with various epitope binding sites may be helpful in resolving this issue. Recently, Higuchi and Aggarwal (1994) have shown that p55 alone is sufficient to induce cytotoxicity, whereas DNA fragmentation requires the involvement of both receptors. Based on their finding, it remains obscure as

to how p75 co-operates with p55 to mediate apoptosis, since by deletion mutant studies, it has been shown that p55 already contains an 80 amino acid cytoplasmic sequence responsible for signaling apoptosis (Tartaglia et al., 1993). Interestingly, this domain also bears 28% homology to the Fas antigen, known to mediate apoptosis (Owen-Schaub et al., 1992). Since the intracellular domain of TNF-R p75 is distinct from p55, it remains to be determined whether a novel death domain is contained within p75, or if p75 assists in apoptosis via some other means, such as recruiting TNF-α to the p55 TNF-R (Tartaglia and Goeddel, 1992) since p75 binds TNF-α with higher affinity than does p55 (Fiers et al., 1991).

In situ hybridization and immunostaining studies have shown that both TNF-Rs are expressed on human villous trophoblast, with p55 present at higher levels (Yelavarthi and Hunt, 1993). Preliminary experiments in this laboratory by flow cytometry have shown that cultured trophoblasts also express both receptors. Other classes of TNF binding sites with K_d in the range of 20-28 nM have also been reported (Eades, Cornelius and Pekela, 1988; Hampson, McLaughlin and Johnson, 1993) and they may also assist in the action of p55 TNF-Rs. My result that the TNF-α mutant which binds exclusively to p55 does not fully reproduce the effects as wild type TNF-α (Figure 6.2) seems to suggest this is the case. It is also interesting to note that p55 transduces the proliferation signals in choriocarcinomas (Yang et al., 1993). The differing differentiation states of choriocarcinoma cell lines and primary villous cytotrophoblasts may dictate different responses to the same signal transduction events, just as primary monocytes survive in the presence TNF-α (Mangan and Wahl, 1991) whereas the monocytic U937 cell line undergoes apoptotic death upon TNF exposure (Jarvis et al., 1994).

A major involvement of NO in the cytotoxicity pathway can be excluded because neither inhibitors of NO synthesis nor the withdrawal of L-arginine blocked TNF-α-induced cytotoxicity in trophoblasts (Table 6.2 and 6.3). In macrophages, it has been shown that the generation of NO by the inducible NO synthase contributes to the apoptotic death of these cells, since this process can be abolished in the presence of NMLA, a NO synthase inhibitor, and can be induced by treating the cells with authentic NO gas (Albina et al., 1993). Whether the inducible form of NO synthase is expressed in CTs and if its expression is upregulated by TNF-α and IFN-γ is not known, although the endothelial constitutive NO synthase is found to be absent in CTs (Buttery et al., 1994).

The upregulation of ROIs upon TNF-α and IFN-γ stimulation contributes to the cytotoxic effects of these cytokines in pancreatic islet cells (Sumoski, Baquerizo and Rabinovitch, 1992). Increased levels of ROIs also induce apoptosis in neural cells, which can be prevented by overexpression of bcl-2, a 25-kd protein capable of prolonging the survival of various B- and T-lymphoid cells via inhibiting apoptotic cell death (Korsmeyer, 1992). Although the exact function of bcl-2 is not known, transfection of the bcl-2 gene rescues neural cell death by downregulating the levels of ROIs, suggestive of a radical-scavenging function for this protein (Kane et al., 1993). Since among fetal tissues, both the ST and CTs stained intensely for the bcl-2 protein (LeBrun, Warnke and Cleary, 1993), this could likely account for the observation that trophoblasts are refractory to the influence of TNF-α and IFN-γ on the upregulation of ROIs (Table 6.4).

In this study, I also showed that trophoblasts were extremely sensitive to the transcriptional inhibitor act D and the translational inhibitor CHX at concentrations above 4 ng/ml and 300 ng/ml respectively; levels which can be

tolerated by other cells without demonstrable toxic effects (Hollenbach, Zilli and Laster, 1992). This suggests that the apoptotic machinery is present constitutively in trophoblasts but under normal circumstances, is suppressed by factors with short half-lives. The observation that TNF-α-induced cytotoxicity is considerably increased in trophoblasts in the presence of act D and CHX is also in accordance with the notion that cytotoxicity induced by TNF-α does not require RNA or protein synthesis (Beyaert and Fiers, 1994). This is in contrast to cell death in thymocytes triggered by anti-CD3 antibodies in which apoptosis requires the synthesis of new proteins and death is therefore blocked in the presence of act D and CHX (Shi et al., 1990). The difference in the mechanisms of cell death between primary epithelial trophoblasts and mesenchymal lymphocytes can be further extended to include their response towards glucocorticoids, as these steroids induce apoptosis in thymocytes and T-cell leukemic lines (Cohen and Duke, 1984) whereas they protect trophoblasts from the toxic effects of TNF-α (Figure 7.1).

E. Antagonism of TNF-α induced cytotoxicity by EGF and glucocorticoids.

The ability of TNF-α to induce the apoptotic death of trophoblasts suggests that other factors may be present at the local placental cytokine network to maintain a balance should there be an upregulated expression of TNF-α at an inappropriate stage of gestation. In chapter VII, I reported that dexamethasone (dex) and EGF inhibited the cytotoxicity induced by TNF-α alone and in combination with IFN-γ (Figure 7.1 and Table 7.1). Both

substances were found to decrease the number of apoptotic nuclei in trophoblasts cultured in the presence of TNF-α and IFN-γ (Figure 7.3).

The question of how glucocorticoids such as dex inhibit the cytotoxic activity of TNF remains a puzzle. It has been proposed that dex induces the synthesis of lipocortin, which subsequently inhibits the activity of PLA_2 (Goulding and Guyre, 1992). In some cell lines, the activation of PLA_2 leads to TNF cytotoxicity (Hayakawa et al., 1993). Upregulation of PLA_2 activity can be achieved by an increase in ceramide levels resulting from the hydrolysis of sphingomyelin, which constitutes one of the TNF-induced post-receptor signaling pathway (Kolesnick and Golde, 1994). However, other researchers have shown that the PLA_2 inhibitory abilities of glucocorticoids are lipocortin-independent (Beyaert et al., 1990); rather, dex directly suppresses TNF-induced PLA_2 production at the post-transcriptional level (Nakano et al., 1990).

The inhibitory effect of dex on TNF-α induced cytotoxicity is transcription-dependent as it requires a minimum of 4 hour pre-incubation with the cells and has been reported to be lost in the presence of act D (Suffys et al., 1987). On the other hand, EGF exerts its inhibitory effect on TNF-induced trophoblast cultures without a prolonged pre-incubation period. This could be accounted for by the fact that the receptor for EGF is an intrinsic tyrosine kinase and upon EGF binding, can immediately phosphorylate other proteins. On the other hand, the biological effects of dex requires its initial binding to a cytosolic receptor, and subsequently the hormone-receptor complex translocates to the nucleus to interact with promoter elements and affect gene transcription. This latter mechanism may take longer and can be subject to control at more steps along the pathway.

The nature of the cellular response which protects trophoblasts against the cytotoxic effects of TNF-α remains a matter of speculation. It is also not known whether dex and EGF induce the same set of protective proteins. In cells where ROIs constitute the primary pathway of TNF-killing, the expression of manganous superoxide dismutase confers protection but this appears an unlikely candidate in trophoblasts, since ROIs are not important mediators in transducing the cytotoxic signal (Table 6.4). Other protective proteins known to counteract the cytotoxic effects of TNF-α include plasminogen activator inhibitor type 2 (PAI-2; Kumar and Baglioni, 1991) which has been documented to be present in trophoblasts (Feinberg et al., 1989; Hofmann et al., 1994), heat shock protein (hsp) 70 (Jaattela et al., 1992) and A20 zinc finger proteins (Opipari et al., 1992). The bcl-2 protein, despite its action in reversing apoptosis, yields controversial results regarding to its capacity in opposing the cytotoxic effects of TNF-α (Opipari et al., 1992; Itoh, Tsujimoto and Nagata, 1993).

F. Proposal of a model for TNF-α activity in placental development.

What is the physiological significance of TNF-α expression in the placenta, given its ability to induce apoptosis in trophoblasts? I propose that under normal conditions of placental development, the regulated levels of TNF-α produced locally at the placenta serves to maintain appropriate numbers of CTs sufficient for the changing needs of the fetus as pregnancy progresses towards term. In situ hybridization and immunohistochemical staining studies have shown that during pregnancy, the expression of TNF-α mRNA and protein gradually increases towards parturition and concomitantly, there is a gradual reduction in the number of CTs. This

selective depletion of CTs could occur to meet the nutritional and metabolic needs for the growing fetus as the process permits the fetal endothelial cells to come closely apposed to the ST by elimination of the middle layer of CTs. This process would facilitate the exchange of gases and nutrients across the ST barrier. Indeed, an examination of placental sections at term reveal the prevalence of these sites known as epithelial plates or vasculosyncytial membranes and their numbers are higher in term than in first trimester placentas (Benirschke and Kaufmann, 1990).

Natural cell death during the course of developmental changes and tissue homeostasis is often achieved via apoptosis because in this process, the plasma membrane of dying cells remains intact and intracellular contents do not leak into the extracellular milieu to initiate inflammatory responses. This is often in stark contrast to necrotic cell death triggered by pathological insult. In addition, apoptosis affects single cells rather than the mass, so regulation can be finely targeted on an individual cell basis. Apoptotic cells are quickly ingested by macrophages, or neighboring sister cells, or they simply sloughed off from the basement membrane. Thus it is not surprising that many physiological events which require the removal of unwanted cells, such as the regression of lactating mammary glands after weaning (Walker, Bennett and Kerr, 1989) and the deletion of autoreactive T-cell clones in the thymus (Shi, Sahai and Green, 1989) resort to the process of apoptosis. It is therefore likely that the elimination of extra CTs also utilize this process.

On the other hand, ascending infections could trigger villous stromal cells, in particular the fetal Hofbauer macrophages, to release TNF-α at high levels, and IFN-γ is also likely to be present under these circumstances. This aberrant expression could be essentially harmful during the early trimesters of pregnancy, since it results in a premature depletion of an underlying pool of

healthy CTs, the precursors from which the syncytium is derived (Benirschke and Kaufmann, 1990). This process could hinder the expansion and maturation of syncytial layer, result in stunted growth of the placenta, and lead to intrauterine growth retardation (IUGR) of the fetus, since placental size closely mirrors fetal growth and development.

Our result that the ST is also sensitive to high concentrations of TNF-α and IFN-γ also warrants consideration of the potential consequences of the release of these pro-inflammatory cytokines at the apical surface of the syncytial barrier during episodes of hematogenous infections. Maternal leukocytes, which can be activated to synthesize TNF-α and IFN-γ after encountering infectious agents, may home to the ST, and via the focal release of these cytokines, introduce microscopic lesions in the barrier. This has important implications as transplacentally transmitted infectious agents, such as HIV, could gain initial access to the fetal circulation via these interruptions in the trophoblastic epithelium, thereby circumventing the need for specific receptors on the ST. In the light of the apparent lack of CD4 expression on trophoblasts (Lairmore et al., 1993), there are at least two reports documenting that focal inflammation of the placenta constitutes the placental lesion that best correlates with the vertical transmission of HIV (Chandwani et al., 1991; St. Louis et al., 1993)

Based on the results which showed that EGF and glucocorticosteroids can directly antagonize the cytotoxicity of TNF-α and IFN-γ, I therefore propose that the placenta has built in defense mechanisms against the excessive and untimely production of pro-inflammatory cytokines. Thus EGF can either directly counteract the cytotoxic effects of TNF-α, or it can stimulate repair of existing lesions in the trophoblastic epithelium by inducing the fusion of a reserved pool of undifferentiated CTs, thereby maintaining the

barrier functions of the placenta. Indeed, lower than average EGF concentrations in pregnancy has been associated with delivery of IUGR infants (Hofmann et al., 1988). Other cytokines that promote cytotrophoblastic differentiation into ST, such as GM-CSF and CSF-1 (Garcia-Lloret et al., in press) may also be beneficial in counteracting TNF effects. The abundance of TGF-βs in the ST and CTs may also contribute to downregulate the cytotoxic effects of TNF-α as TGF-βs are known for their immunosuppressive activities against inflammatory responses (Palladino et al., 1990; Wahl et al., 1990).

Another mechanism of protection against damage caused by excessive TNF-α and IFN-γ during pregnancy is to shift the immunity at the fetal-maternal interface to a humoral biased response, characterized by the production of Th2 type cytokines like IL-4, IL-5 and IL-10 in order to dampen the effects of pro-inflammatory cytokines, which belong to the Th1 pattern. In mice, this bias towards Th2 cytokine production in reproductive tissues is supported by experimental evidence (Hui et al., 1993). Although in humans, the existence of such a pattern is yet undefined, clinical observations that autoimmune diseases characterized by antibody production (likely regulated by Th2 cells) such as systemic lupus tend to worsen during pregnancy whereas those involving cell-mediated immunity (regulated by Th1 cells) such as rheumatoid arthritis show improvements suggests a likelihood of this bias (Wegmann et al., 1993 and references therein). Furthermore, the sustained high levels of glucocorticosteroid production during pregnancy also serve to steer the response towards a Th2 like pattern (Rook, Hernandez-Pando and Lightman, 1994). Thus the cytokine-endocrine network in the placenta is established to maintain the appropriate levels of TNF-α during

gestation and to strike a balance of TNF-α levels with other cytokines at the maternal-fetal interface.

A third mechanism of regulating TNF-α activity is through the shedding of soluble TNF receptors. In this study, p55 has been shown to be the major receptor in mediating the cytotoxicity in trophoblasts. This same receptor in soluble form has been reported to be present in increasing levels in pregnant women as gestation proceeds (Austgulen et al., 1992). The shedding of these receptors may serve to neutralize excess TNF activity and to prevent the accumulation of TNF-α as this cytokine also increases towards term due to an increased capacity of placental macrophages to transcribe the gene (Chen et al., 1991). Interestingly, a sudden reduction in soluble TNF-Rs is detected just before parturition, and this is proposed to initiate uterine contractions and induce delivery (Austgulen et al., 1992).

A summary of the proposed model is depicted in Figure 8.1. It has to be remembered that synthesis of TNF-α is not confined to villous trophoblasts; other cells at the uteroplacental unit also transcribe and translate the TNF-α gene in a gestation-dependent manner (Chen et al., 1991). Thus apart from the role in villous remodeling as suggested in this model here, TNF-α has also been proposed to promote angiogenesis, the synthesis of collagenase, to limit the extent of extravillous trophoblast invasion into the uterine wall, and to downregulate HLA expression in embryonic fibroblasts. Unraveling the multifunctional aspects of TNF-α on placental cells other than trophoblasts should provide a more detailed understanding on cytokine regulation during placental development.

G. Future Directions

The suggested model predicts that an upregulation of TNF-α levels, either due to increased production of the cytokine as a result of ascending infections, or due to a failure in control mechanisms, is harmful to placental development. The verification of this model will require examination of clinical specimens from both normal and diseased placentas, the latter, for instance obtained from IUGR fetus or from those associated with transplacental infections. The localization of TNF-α expression in normal *vs* abnormal situations could be determined by immunohistochemical staining or *in situ* hybridization. TNF-α in the amniotic fluid or placental cell culture supernatants may be used as an indicator of TNF-α levels *in vivo*. While doing this, one has to keep in mind that the proposed model emphasizes a balance of cytokine production in normal pregnancy, thus the relative amounts of other cytokines and modulators of TNF actions, such as EGF, IL-10, TGF-β, GM-CSF, CSF-1, soluble TNF-Rs and steroids should be compared to those of TNF-α.

It is also important to determine whether TNF-α expression correlates with apoptosis *in vivo*. The TUNEL method can be equally well applied to examining tissues on paraffin or frozen placental sections. The co-localization of TUNEL positive cells in the neighborhood of positive TNF-α staining would be in favor of such a process occurring *in vivo*.

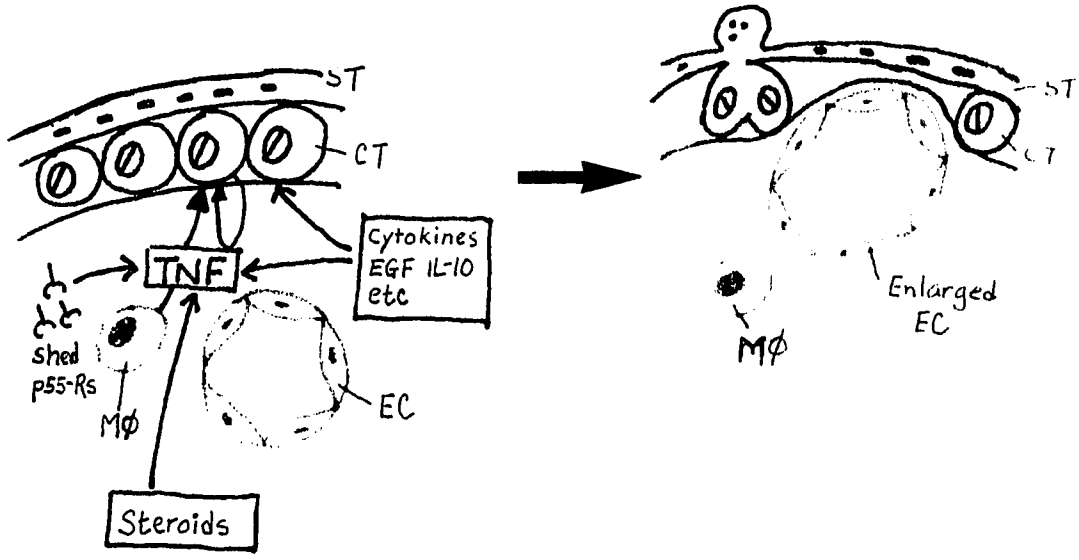
If aberrant expression of TNF-α does contribute to pathological situations and if these harmful effects are downregulated by cytokines and glucocorticosteroids, then understanding the mechanism of downregulation may help in designing effective strategies to overcome harmful TNF effects.

What cell types in the placenta are producing the beneficial cytokines? Can their production be manipulated pharmacologically? What is the nature of the intracellular response to protective cytokines and steroids?

The preliminary work presented in this thesis not only generates these questions but also suggests some experimental approaches. For example, I have repeatedly shown that in primary cultures, there always exists a population of cells which are inherently resistant to the TNF-α cytotoxic effects. If sensitive and resistant populations can be sorted and their properties analyzed, they may serve as useful models to provide answers to the above questions. Are the levels of p55 and p75 expressed on these two populations different? Do the resistant cells constitutively produce high levels of EGF, IL-10 and/or soluble TNF-Rs? Alternatively, transformation of these primary cells into 100% resistant or 100% sensitive to TNF killing may be useful in studying the mechanisms of TNF cytotoxic action. Clearly, this study reported here opens up more interesting questions to be addressed before we fully understand the functions of TNF-α *in vivo*.

Figure 8.1

A. Normal



B. Aberrant

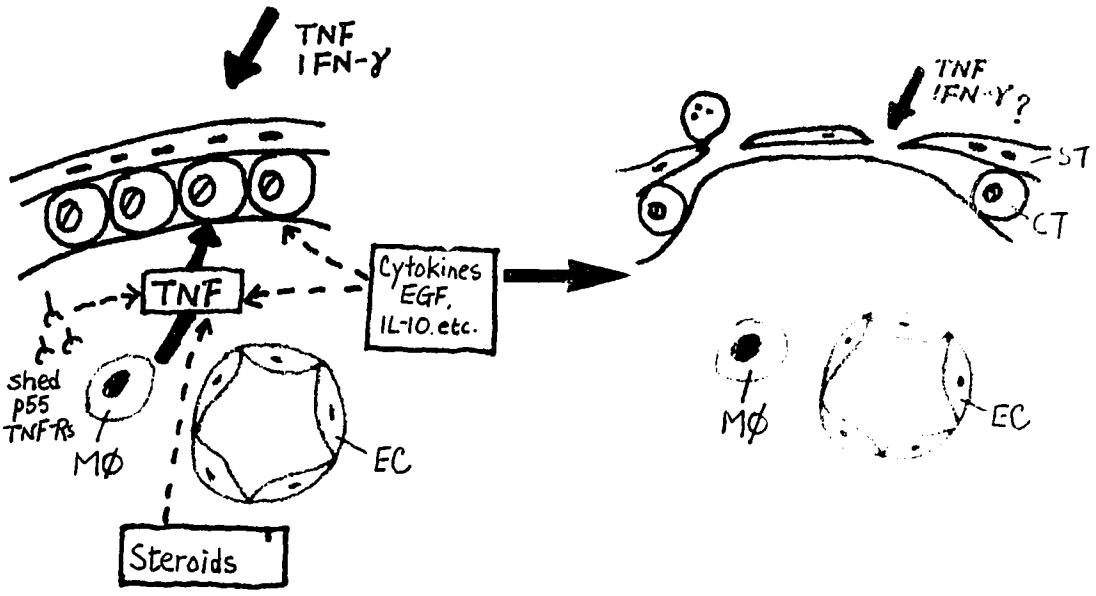


Figure 8.1. A hypothetical model for the function of TNF-α in placental development. (A) Under normal situation, when TNF-α levels are regulated, the gradual increase in TNF-α expression as pregnancy progresses towards term serves to deplete CTs at the appropriate timing and location to allow enlarging sinusoids to bulge against the syncytial surface and thereby facilitate gaseous exchange. Any surge of TNF-α would be downregulated by the presence of steroids, cytokines such as EGF, IL-10, TGF-β and by the shedding of TNF-Rs, particularly p55. Although aged nuclei of the ST cluster into knots and later pinch off from the surface, the fusion of the underlying CTs to regenerate the ST helps maintain the continuity of the syncytial barrier. Red arrows: cellular sources of TNF; black arrows: biological mediators with the potential to counteract TNF effects. A balance of these two is indicated by arrows of the same thickness.

(B) In an aberrant situation, where control mechanisms have failed, the enhanced levels of TNF-α, particularly in combination with IFN-γ, serves to deplete the pool of CTs required for fusion into the ST. If this process occurs during a time of syncytial growth, it could lead to small placentas as in IUGR. In addition, the process of replenishing aging ST by newly fused CTs is compromised, and may lead to loss of structural integrity of the barrier. TNF-α and IFN-γ arising from the maternal side may also directly damage the ST. Thick arrows: upregulated expression; dotted arrows: decreased expression relative to that which is sufficient to suppress the cytotoxic effects of TNF-α and IFN-γ.

CHAPTER IX
BIBLIOGRAPHY

Aboagye-Mathiesen, G., Toth, F.D., Peterson, P.M., Gildberg, A., Norskov-Lauritsen, N., Zachar, V. & Ebbesen, P. (1993) Differential interferon production in human first and third trimester trophoblast cultures stimulated with viruses. *Placenta*, 14, 225-234.

Aggarwal, B.B., Eessalu, T.E. & Hass, P.E. (1985). Characterization of receptors for human tumor necrosis factor and their regulation by γ interferon. *Nature*, 318, 665-668.

Albina, J.E., Cui, S., Mateo, R.B., Reichner, J.S. (1993) Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *The Journal of Immunology*, 150, 5080-5085.

Anderson, D.J. & Berkowitz, R.S. (1985) γ-interferon enhances expression of class I MHC antigens in the weakly HLA^+ human choriocarcinoma cell line BeWo, but does not induce MHC expression in the HLA^-choriocarcinoma cell line Jar. *The Journal of Immunology*, 135, 2498-2501.

Andrews, P.W., Knowles, B.B. & Goodfellow, P.N. (1981) A human cell-surface antigen defined by a monoclonal antibody and controlled by a gene on chormosome 12. *Somatic Cell Genetics*, 7, 435-443.

Androlewicz, M.J., Browning, J.L. & Ware, C.F. (1992) Lymphotoxin is expressed as a heteromeric complex with a distinct 33-kd glycoprotein on the surface of an activated human T cell hybridoma. *The Journal of Biological Chemistry*, 267, 2542-2547.

Aplin, J.D. (1991) Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence *in vivo* and *in vitro*. *Journal of Cell Science*, 99, 681-692.

Areci, R.J., Shanahan, F., Stanley, E.R. & Pollard, J.W. (1989) Temporal expression and location of colony-stimulating factor 1 (CSF-1) and its receptors in the female reproductive tract are consistent with CSF-1-regulated placental development. *Proceedings of the National Academy of Sciences, USA*, 86, 8818-8822.

Arends, M.J., Morris, R.G. & Wyllie, A.H. (1990) Apoptosis: the role of the endonuclease. *American Journal of Pathology*, 136, 593-608.

Armstrong, D.T. & Chaouat, G. (1989) Effects of lymphokines and immune complexes on murine placental cell growth in vitro. *Biology of Reproduction*, 40, 466-474.

Athanassakis, I., Bleackley, C., Paetkau, V., Guilbert, L.J., Parr, P.J. & Wegmann, T.G. (1987) The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells. *The Journal of Immunology*, 138, 37-41.

Austgulen, R., Liabakk, N-B., Brockhaus, M. & Espevik, T. (1992) Soluble TNF receptors in amniotic fluid and in urine from pregnant women. *Journal of Reproductive Immunology*, 22, 105-116.

Austgulen, R., Liabakk, N-B., Lien, E. & Espevik, T. (1993) Increased levels of soluble tumor necrosis factor-α receptors in serum from pregnant women and in serum and urine samples from newborns. *Pediatric Research*, 33, 82-87.

Autio-Harmainen, H., Snadbert, M., Philajaniemi, T.& Vuorio, E. (1991) Synthesis of laminin and type IV collagen by trophoblastic cells and fiboblastic stromal cells in the early human placenta. *Laboratory Investigation*, 64, 483-491.

Awater, J.A., Wisdom, R. & Verma, I.M. (1990) Regulated mRNA stability. *Annual Review in Genetics*, 24, 519-541.

Banner, D.W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H-J., Broger, C., Loetscher, H. & Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNFβ complex: implications for TNF receptor activation. *Cell*, 73, 431-445.

Barnea, E.R., Feldman, D., Kaplan, M. & Morrish, D.W. (1990) The dual effect of epidermal growth factor upon human chorionic gonadotropin secretion by the first trimester placenta in vitro. *Journal of Clinical Endocrinology and Metabolism*, 71, 923-928.

Bartocci, A., Pollard, J.W.& Stanley, E.R. (1986) Regulation of colony-stimulating factor 1 during pregnancy. *Journal of Experimental Medicine*, 164, 956-961.

Bass, K.E., Morrish, D., Roth, I., Bhardwaj, D., Taylor, R., Zhou, Y. & Fisher, S.J. Human cytotrophoblast invasion is upregulated by epidermal growth factor: evidence that paracrine factors modify this process. *Developmental Biology*, in press.

Beelen, R.H.J., Bos, H.J., Kamperdijk, E.W.A. & Hoefsmit, E.C.M. (1989) Ultrastructure of monocytes and macrophages. In *Human Monocytes* (Ed.) Zembala, M. & Asherson, G.L. pp. 7-16. London: Academic Press.

Benirschke, K. & Kaufmann, P. (1990) Pathology of the Human Placenta. pp 22-70. New York: Springer-Verlag.

Berkowitz, R.S., Hill, J.A., Kurtz, C.B. & Anderson, D.J. (1988) Effects of products of activated leukocytes (lymphokines and monokines) on the growth of malignant trophoblast cells in vitro. *American Journal of Obstetrics and Gynecology*, 158, 199-203.

Berkowitz, R.S., Faris, H. M. P., Hill, J.A. & Anderson, D.J. (1990) Localization of leukocytes and cytokines in chorionic villi of normal placentas and complete hydatidiform moles. *Gynecologic Oncology*, 37, 396-400.

Ber ni, R., Bianchi, M.& Ghezzi, P. (1988) Adrenalectomy sensitizes mice to ne lethal effects of interleukin 1 and tumor necrosis factor. *The Journal of Experimental Medicine*, 167, 1708-1712.

Beutler, B. & Cerami, A. (1989) The biology of cachetin /TNF-α primary mediator of the host response. *Annual Review of Immunology*, 7, 625-655.

Beutler, B. & van Huffel, C. (1994) Unraveling function in the TNF ligand and receptor families. *Science*, 264, 667-668.

Beyaert, R., Suffys, P., Van Roy, F. & Fiers, W. (1990) Inhibition by glucocorticoids of tumor necrosis factor-mediated cytotoxicity. Evidence against lipocortin involvement. *FEBS*, 262, 93-96.

Billard, C. Signaux, F. & Wietzerbin, J. (1990) IFN-α in vivo enhances tumor necrosis factor receptor levels on hairy cells. *The Journal of Immunology*, 145, 1713-1718.

Bischof, P., Friedli, E., Martelli, M. & Campana, A. (1991) Expression of extracellular matrix-degrading metalloproteinases by cultured human cytotrophoblast cells: effects of cell adhesion and immunopurification. *American Journal of Obstetrics and Gynecology*, 165, 1791-1801.

Boehm, K.D., Kelley, M.F., Ilan, J. & Ilan, J. (1989) The interleukin 2 gene is expressed in the syncytiotrophoblast of the human placenta. *Proceedings of the National Academy of Sciences, USA*, 86, 656-660.

Boyd, J.D. & Hamilton, W.J. (1970) The Human Placenta. Cambridge: W. Heffer and Sons, pp1-26, 140-173.

Brady, G., Barbara, M. & Iscove, N.N. (1990) Representative in vitro cDNA amplification from individual hemopoietic cells and colonies. *Methods in Molecular and Cellular Biology*, 2, 17-25.

Branca, A.A. (1986) High-affinity receptors from human interferon in bovine and human placenta. *Journal of Interferon Research*, 6, 305-311.

Bredt, D.S. & Snyder, S.H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proceedings of the National Academy of Sciences, USA*, 87, 682-685.

Brockhaus, M., Schoenfeld, H-J., Schlager, E.J., Hunziker, W. Lesslauer, W & Loetscher, H. (1990) Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proceedings of the National Academy of Sciences, USA*, 87, 3127-3131.

Browning, J.L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E.P., Hession, C., O'Brine-Greco, B., Foley, S.F. & Ware, C.F. (1993) Lymphotoxin β, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell*, 72, 847-856.

Brouckaert, P., Everaerdt, B. & Fiers, W. (1992) The glucocorticoid antagonist RU38486 mimics interleukin-1 in its sensitization to the lethal

and interleukin-6-inducing properties of tumor necrosis factor. *European Journal of Immunology*, 22, 981-986.

Bullen, B.E., Bloxam, D.L., Ryder, T.A., Mobberley, M.A. & Bax, C.M.R. (1990) Two-sided culture of human placental trophoblast. Morphology, immunocytochemistry and permeability properties. *Placenta*, 11, 431-450.

Bulmer , J.N. & Johnson, P.M. (1984) Macrophage populations in the human placenta and amniochorion. *Clinical and Experimental Immunology*, 57, 393-403.

Bulmer, J.N., Morrison, L., Johnson, P.M. & Meager, A. (1990) Immunohistochemical localization of interferons in human placental tissues in normal, ectopic, amd molar pregnancy. *American Journal of Reproductive Immunol*ogy, 22, 109-116.

Burgess, A.W., Wilson, E.M.A. & Metcalf, D. (1977) Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. *Blood*, 49, 573-583.

Buttery, L.D.K., McCarthy, A., Springall, D.R., Sullivan, M.H.F., Elder, M.G., Michel, T. & Polak, J.M. (1994) Endothelial nitric oxide synthase in the human placenta: regional distribution and proposed regulatory role at the feto-maternal interface. *Placenta*, 15, 257-265.

Byrkit, D.R. (1987) Statistics Today: A Comprehensive Introduction. pp.687-689. Menlo Park: The Benjamin/ Cummings Publishing Company, Inc.

Camussi, G., Albanao, E., Tetta, C. & Bussolino, F. (1991) The molecular action of tumor necrosis factor-α. *European Journal of Biochemistry*, 202, 3-14.

Capon, D.J. & Ward, R.H. (1991) The CD4-gp120 interaction and AIDS pathogenesis. *Annual Review of Immunology*, 9, 649-678.

Carpenter, G. & Cohen, S. (1979) Epidermal growth factor. *Annual Review of Biochemistry*, 48, 193-216.

Carson , D.A. & Ribeiro, J.M. (1993) Apoptosis and disease. *The Lancet*, 341, 1251-1254.

Carson, S.A., Chase, R., Ulep, E., Scommegna, A. & Benveniste, R. (1983) Ontogenesis and characteristics of epidermal growth factor receptors in human placenta. *American Journal of Obstetrics and Gynecology,* 147, 932-939.

Casey, M.L., Cox, S.M., Beutler, B., Milewich, L. & MacDonald, P.C. (1989) Cachetin/ tumor necrosis factor-α formation in human decidua. Potential role of cytokines in infection-induced preterm labor. *Journal of Clinical Investigation,* 83, 430-436.

Castellucci, M., Celona, A., Bartels, H., Steininger, B., Benedetto, V. & Kaufmann, P. (1987) Mitosis of Hofbauer cell: possible implications for a fetal macrophage. *Placenta,* 8, 65-76.

Cesarone, C.F., Bolognesi, C. & Santi, L. (1979) Improved microfluorometric DNA determination in biological material using 33248 Hoechst. *Analytical Biochemistry,* 100, 188-197.

Chandwani, S., Greco, M.A., Mittal, K., Antoine, C., Krasinski, K. & Borkowsky, W. (1991) Pathology and human immunodeficiency virus expression in placentas of seropositive women. *The Journal of Infectious Diseases,* 163, 1134-1138.

Chang, M-D Y., Pollard, J.W., Khalili, H., Goyert, S.M. & Diamond, B. (1993) Mouse placental macrophages have a decreased ability to present antigen. *Proceedings of the National Academy of Sciences, USA,* 90, 462-466.

Chaouat, G., Menu, E., Clark, D.A., Dy, M., Minkowski, M. & Wegmann, T.G. (1990) Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. *Journal of Reproduction and Fertility,* 89, 447- 458.

Chegini, N. and Rao, Ch.V. (1985) Epidermal growth factor binding to human amnion, chorion, decidua, and placenta from mid-and term pregnancy: quantitative light microscopic autoradiographic studies. *Journal of Clinical Endocrinology and Metabolism,* 61, 529-535.

Chen, C-F, Kurachi, H., Fujita, Y., Terakawa, N., Miyake, A. & Tanizawa, O. (1988) Changes in epidermal growth factor receptor and its messenger ribonucleic acid levels in human placenta and isolated trophoblast cells

during pregnancy. *Journal of Clinical Endocrinology and Metabolism*, 67, 1171-1177.

Chen, H-L., Yang,Y., Hu, X-L., Yelavarthi, K.K., Fishback, J.L. & Hunt, J.S. (1991) Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. *American Journal of Pathology*, 139, 327-335.

Clark, S.C. & Kamen, R. (1987) The human hematopoietic colony-stimulating factors. *Science*, 236, 1229-1237.

Cohen, J.J. 1993. Apoptosis. *Immunology Today*, 14, 126-130.

Cohen, J.J. & Duke, R.C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *The Journal of Immunology*, 132, 38-42.

Cohen, S., Pick, E. & Oppenheim, J.J. (1979) In *Biology of Lymphokines*, Pick, E. & Oppenheim, J.J. (Eds). New York: Academic Press, pp 1-7.

Contractor, S.F., Banks, R.W., Jones, C.J.P. & Fox, H. (1977) A possible role for placental lysosomes in the formation of villous syncytio-trophoblast. *Cell and Tissue Research*, 178, 411-419.

Corbett, J.A., Sweetland, M.A., Wang, J.L., Lancaster, Jr., J.R. & McDaniel, M.L. (1993) Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proceedings of the National Academy of Sciences, USA*, 90, 1731-1735.

Cotte, C. Easty, G.C., Neville, A.M. & Monaghan, P. (1980) Preparation of highly purified cytotrophoblast from human placenta with subsequent modulation to form syncytiotrophoblast in monolayer cultures. *In Vitro*, 16, 639-646.

Crissman, H.A. and Steinkamp, J.A. (1973) Rapid simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. *Journal of Cell Biology*, 59, 766-771.

Crowe, P.D., VanArsdale, T.L., Walter, B.N., Ware, C.F., Hession, C., Ehrenfels, B., Browning, J.L., Din, W.S., Goodwin, R.G.& Smith, G.A. (1994) A lymphotoxin-β-specific receptor. *Science*, 264, 707-710.

Curran, R.D, Billiar, T.R., Stuehr, D.J., Hofmann, K., Simmons, R.L. (1989) Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. *The Journal of Experimental Medicine*, 170, 1769-1774.

Daiter E., Pampfer, S., Yeung, Y.G., Barad, D., Stanley, E.R. & Pollard, J.W. (1992) Expression of colony-stimulating factor-1 in the human uterus and placenta. *Journal of Clinical Endocrinology and Metabolism*, 74, 850-858.

Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A. & Stewart, T.A. (1993) Multiple defects of immune cell function in mice with disrupted interferon-γ genes. *Science*, 259, 1739-1742.

Damsky, C.H., Fitzgerald, M.L. & Fisher, S.J. (1992) Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *Journal of Clinical Investigation*, 89, 210-222.

Damsky, C., Sutherland, A. & Fisher, S. (1993) Adhesive interactions in early mammalian embryogenesis, implantation, and placentation. *FASEB J.* 7, 1320-1329.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G. & de Vries, J.E. (1991) Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *Journal of Experimental Medicine*, 1209-1220.

Dempsey, E.W. (1972) The development of capillaries in the villi of early human placentas. *American Journal of Anatomy*, 134, 221-238.

Deng, W., Thiel, B., Tannenbaum, C.S., Hamilton, T.A. & Stuehr, D.J. (1993) Synergistic cooperation between T cell lymphokines for induction of the nitric oxide synthase gene in murine peritoneal macrophages. *The Journal of Immunology*, 151, 322-329.

Dennis, E.A., Rhee, S.G., Billah, M.M. & Hannun, Y.A. (1991) Role of phospholipases in generating lipid second messengers in signal transduction. *FASEB Journal*, 5, 2068-2077.

Dinarello, C.A. (1988) Biology of interleukin 1. *FASEB Journal*, 2, 108-115.

Dinarello, C.A. (1993) Modalities for reducing interleukin 1 activity in disease. *Immunology Today*, 14, 260-264.

Dinarello, C.A. & Thompson, R.C. (1991) Blocking IL-1: interleukin 1 receptor antagonist *in vivo* and *in vitro*. *Immunology Today*, 12, 404-410.

DiPersio, J., Billing, P., Kaufman, S., Eghtesady, P., Williams, R.E. & Gasson, J.C. (1988) Characterization of the human granulocyte-macrophage colony-stimulating factor receptor. *The Journal of Biological Chemistry*, 263, 1834-1841.

Douglas, G.C. and King, B.F. (1989) Isolation of pure villous cytotrophoblast from term human placenta using immunomagnetic microspheres. *Journal of Immunological Methods*, 119, 259-268.

Douglas, G.C. and King, B.F. (1990) Differentiation of human trophoblast cells in vitro as revealed by immunocytochemical staining of desmoplakin and nuclei. *Journal of Cell Science*, 96, 131-141.

Douglas, D.C. & King, B.F. (1992) Maternal-fetal transmission of human immunodeficiency virus: a review of possible routes and cellular mechanisms of infection. *Clinical Infectious Diseases*, 15, 678-691.

Downward, J., Yarden, Y., Mayer, E., Scarce, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. & M.D. Waterfield. (1984) Close similarity of the epidermal growth factor receptor and the *v-erbB* oncogene nucleotide sequences. *Nature*, 307, 521-527.

Drake, B.L. & Head, J.R. (1990) Murine trophoblast cells are not killed by tumor necrosis factor-α. *Journal of Reproductive Immunology*, 17, 93-99.

Dranoff, G., Crawford, A.D., Sadelain, M., Ream, B., Rashid, A., Bronson, R.T., Dickersin, G.R., Bachurski, C.J., Mark, E.L., Whitsett, J.A. & Mulligan, R.C. (1994) Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science*, 264, 713-716.

Dressler, K.A., Mathias, S. & Kolesnick, R.N. (1992) Tumor necrosis factor-α activates the sphingomyelin signal transduction pathway in a cell-free system. *Science*, 255, 1715-1718.

Eades, D.K., Cornelius, P.& Pekala, P.H. (1988) Characterization of the tumor necrosis factor receptor in human placenta. *Placenta*, 9, 247-251.

Engelmann, H., Aderka, D., Rubinstein, M., Rotman, D. & Wallach, D. (1989) A tumor necrosis factor binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity. *The Journal of Biological Chemistry*, 264, 11974-11980.

Engelmann, H., Holtmann, H., Brakebusch, C., Avni, Y.S., Sarov, I., Nophar, Y., Hadas, E., Leitner, O. & Wallach, D. (1990) Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *The Journal of Biological Chemistry*, 265, 14497-14504.

Espevik, T., Figari, I.S., Shalaby, M.R., Lackides, G.A., Lewis, G.D., Shepard, H.M. & Palladino, Jr., M.A. (1987) Inhibition of cytokine production by cyclosporin A and transforming growth factor β. *The Journal of Experimental Medicine*, 166, 571-576.

Espevik, T., Brockhaus. M., Loetscher, H., Nonstad, U.& Shalaby, R. (1990) Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. *Journal of Experimental Medicine*, 171, 415-426.

Farrar, M.A., Campbell, J.D. & Schreiber, R.D. (1992) Identification of a functionally important sequence in the C terminus of the interferon-γ receptor. *Proceedings of the National Academy of Sciences, USA,* 89, 11706-11710.

Feinberg, R.F., Kao, L-C., Haimowitz, J.E., Queenan, Jr., J.T., Wun, T-C., Strauss III, J.F. & Kliman, H.J. (1989) Plasminogen activator inhibitor types 1 and 2 in human trophoblasts. PAI-1 is an immunocytochemical marker of invading trophoblasts. *Laboratory Investigation*, 61, 20-26.

Feinman, M.A., Kliman, H.J. & Main, E.K. (1987) HLA antigen expression and induction by γ-interferon in cultured human trophoblasts. *American Journal of Obstetrics and Gynecology*, 157, 1429-1434.

Fiers, W. (1991) Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. *FEBS*, 285, 199-212.

Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W. & O'Garra, A. (1991) IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *The Journal of Immunology*, 146, 3444-3451.

Flynn, A., Finke, J.H. & Hilfiker. (1982) Placental mononuclear phagocytes as a source of interleukin-1. *Science*, 218, 475-477.

Forsyth, K.D. (1991) Anti-CD9 antibodies augment neutrophil adherence to endothelium. *Immunology*, 72, 292-296.

Fox, H. (1978) Pathology of the Placenta. pp.1-37, 149-197. Philadelphia: Saunders.

Fulop, V., Steller, M.A., Berkowitz, R.S. & Anderson, D.J. (1992) Interferon-γ receptors on human gestational choriocarcinoma cell lines: quantitative and functional studies. *American Journal of Obstetrics and Gynecology*, 167, 524-530.

Galton, M. (1962) DNA content of placental nuclei. *Journal of Cell Biology*, 13, 183-191.

Garcia-Lloret, M.I., Morrish, D.W., Wegmann, T.G., Honore, L., Turner, A.R. & Guilbert, L.J. Demonstration of functional cytokine-placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion. *Experimental Cell Research*, in press.

Gavrieli, Y., Sherman, Y. & Ben-Sasson, S.A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *The Journal of Cell Biology*, 119, 493-501.

Gazzinelli, R.T., Oswald, I.P., James, S.L. & Sherr, A. (1992) IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. *Journal of Immunology*, 148, 1792-1796.

Gearing, D.P., King, J.A., Gough, N.M. & Nicola, N.A. (1989) Expression cloning of a receptor form human granulocyte-macrophage colony-stimulating factor. *EMBO Journal*, 8, 3667-3676.

Geller, D.A., Lowenstein, C.J., Shapiro, R.A., Nussler, A.K., Di Silvio, M., Wang, S.C., Nakayama, D.K., Simmons, R.L., Snyder, S.H. & Billiar, T.R. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proceedings of the National Academy of Sciences, USA,* 90, 3491-3495.

Gendron, R.L., Nestel, F.P., Lapp, W.S. & Baines, M.G. (1990) Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumor necrosis factor-alpha. *Journal of Reproduction and Fertility*, 90, 395-402.

Gerdes, J., Schwab, U., Lemke, H. & Stein, H. (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *International Journal of Cancer*, 31, 13-20.

Gerschenson, L.E. & Rotello, R.J. (1992) Apoptosis : a different type of cell death. The *FASEB Journal*, 6, 2450-2455.

Goldstein, J., Braverman, M., Salafia, C. & Buckley, P. (1988) The phenotype of human placental macrophages and its variation with gestational age. *American Journal of Pathology*, 133, 648-659.

Goulding, N.J. & Guyre, P.M. (1992) Regulation of inflammation by lipocortin 1. *Immunology Today*, 13, 295-297.

Greenblatt, M.S. & Elias, L. (1992) The type B receptor for tumor necrosis factor-α mediates DNA fragmentation in HL-60 and U937 cells and differentiation in HL-60 cells. *Blood*, 80, 1339-1346.

Gregory, H. (1975) Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature, 257, 325- 327.

Guilbert, L.J., Robertson, S.A. & Wegmann, T.G. (1993) Trophoblast as an integral component of a macrophage-cytokine network. *Immunology and Cell Biology*, 71, 49-57.

Hakoshima, T. & Tomita, K. (1988) Crystallization and preliminary X-ray investigation reveals that tumor necrosis factor is a compact trimer furnished with 3-fold symmetry. *Journal of Molecular Biology*, 201, 455-457.

Hampson, J., McLaughlin, P.J., Johnson, P.M. (1993) Low-affinity receptors for tumor necrosis factor-α, interferon-γ and granulocyte-macrophage colony-stimulating factor are expressed on human placental syncytiotrophoblast. *Immunolgy*, 79, 485-490.

Harty, J.R. & Kauma, S.W. (1992) Interleukin-1β stimulates colony-stimulating factor-1 production in placental villous core mesenchymal cells. *Journal of Clinical Endocrinology and Metabolism*, 75, 947-950.

Hawes, C.S., Suskin, H.A., Petropoulos, A., Latham, S.E. & Mueller, U.W. (1994) A morphologic study of trophoblast isolated from peripheral blood of pregnant women. *American Journal of Obstetrics and Gynecology*, 170, 1297-1300.

Hayashida, K., Kitamura, T., Gorman, D.M., Aria, K., Yokota, T. & Miyajima, A. (1990) Molecular cloning of a second subunit of the receptor for human granuiocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor. *Proceedings of the National Academy of Sciences, USA*, 87, 9655-9659.

Haynes, M.K., Jackson, L.G., Tuan, R.S., Shepley, K.J. & Smith, B. (1993) Cytokine production in first trimester chorionic villi: detection of mRNAs and protein products *in Situ*. *Cellular Immunology*, 151, 300-308.

Heine, U.I., Munoz, E.F., Flanders, K.C., Ellingsworth, L.R., Lam, H-Y.P., Thompson, N.L., Roberts, A.B. & Sporn, M.B. (1987) Role of transforming growth factor-β in the development of the mouse embryo. *The Journal of Cell Biology*, 105, 2861-2876.

Heisterkamp, N., Groffen, J. & Stephenson, J.R. (1983) Isolation of v-fms and its human cellular homolog. *Virology*, 126, 248-258.

Heller, R.A., Song, K., Fan, N. & Chang, D. (1992) The p70 tumor necrosis factor receptor mediates cytotoxicity. *Cell*, 70, 47-56.

Hemmi, S., Merlin, G. & Aguet, M. (1992) Functional characterization of a hybrid human-mouse interferon-γ receptor: Evidence for species-specific interaction of the extracellular receptor domain with a putative signal transducer. *Proceedings of the National Academy of Sciences, USA*, 89, 2737-2741.

Heyborne, K.D., Witkin, S.S. & McGregor, J.A. (1992) Tumor necrosis factor-α in midtrimester amniotic fluid is associated with impaired intrauterine fetal growth. *American Journal of Obstetrics and Gynecology*, 167, 920-925.

Higuchi, M. & Aggarwal, B.B. (1994) Differential roles of two types of TNF receptors in TNF-induced cytotoxicity, DNA fragmentation, and differentiation. *Journal of Immunology*, 152, 4017-4025.

Hill, J.A. (1992) Cytokines considered critical in pregnancy. *American Journal of Reproductive Immunology*, 28, 123-126.

Hill, D.J., Clemmons, D.R., Riley, S.C., Bassett, N. & Challis, J.R. (1993) Immunohistochemical localization of insulin-like growth factors (IGFs) and IGF binding proteins -1, -2 amnd -3 in human placenta and fetal membranes. *Placenta*, 14, 1-12.

Hillier, S.L., Witkin, S.S., Krohn, M.A., Heather Watts, D., Kiviat, N.B. & Eschenbach, D.A. (1993) The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. *Obstetrics and Gynecology*, 81, 941-948.

Hofmann, G.E., Rao, C.V., Brown, M.J., Murray, L.F., Schultz, G.S. & Siddiqi, T.A. (1988) Epidermal growth factor in urine of nonpregnant women and pregnant women throughout pregnancy and at delivery. *Journal of Clinical Endocrinology and Metabolism*, 66, 119-123.

Hofmann, G.E., Scott, R.T., Bergh, P.A. & Deligdisch, L. (1991) Immunohistochemical localization of epidermal growth factor in human endometrium, decidua, and placenta. *Journal of Clinical Endocrinology and Metabolism*, 73, 882-887.

Hofmann, G.E., Glatstein, I., Schatz, F., Heller, D. & Deligdisch, L. (1994) Immunohistochemical localization of urokinase-type plasminogen activator and the plasminogen activator inhibitors 1 and 2 in early human implantation sites. *American Journal of Obstetrics and Gynecology*, 170, 671-676.

Hollenberg, M.D. & Gregory, H. (1977) Human urogastrone and mouse epidermal growth factor share a common receptor site in cultured human fibroblasts. Life Sciences, 20, 267- 274.

Howard, M. & O'Garra, A. (1992) Biological properties of interleukin 10. *Immunology Today*, 13, 198-200.

Hsi, B.L. and Yeh, C.J.G. (1986) Monoclonal antibody GB25 recognizes human villous trophoblasts. *American Journal of Reproductive Immunology and Microbiology*, 12, 1-3.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M. & Aguet, M. (1993) Immune response in mice that lack the interferon-γ receptor. *Science*, 259, 1742-1745.

Hunt, J.S. (1989) Cytokine networks in the uteroplacental unit: macrophages as pivotal regulatory cells. *Journal of Reproductive Immunology*, 16, 1-17.

Hunt, J.S. and Orr, H.T. (1992) HLA and maternal-fetal recognition. *FASEB Journal*, 6, 2344-2348.

Hunt, J.S., King, Jr., C.R. & Wood, G.W. (1984) Evaluation of human chorionic trophoblast cells and placental macrophages as stimulators of maternal lymphocyte proliferation in vitro. *Journal of Reproductive Immunology*, 6, 377-391.

Hunt, J.S., Andrews, G.K. & Wood, G.W. (1987) Normal trophoblasts resist induction of class I HLA. *The Journal of Immunology*, 138, 2481-2487.

Hunt, J.S., Atherton, R.A. & Pace, J.L. (1990) Differential responses of rat trophoblast cells and embryonic fibroblasts to cytokines that regulate proliferation and class I MHC antigen expression. *The Journal of Immunology*, 145, 184-189.

Hunt, J.S., Fishback, J.L., Andrews, G.L. & Wood, G.W. (1988) Expression of class I HLA genes by trophoblast cells. Analysis by in situ hybridization. *The Journal of Immunology*, 140, 1293-1299.

Hunt, J.S., Fishback, J.L., Chumbley, G.& Loke (1990) Identification of class I MHC mRNA in human first trimester trophoblast cells by in situ hybridization. *The Journal of Immunology*, 144, 425.

Hynes, R.O. & Lander, A. D. (1992) Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell*, 68, 303-322.

Idzerda, R.L., March, C.J., Mosley, B., Lyman, S.D., Vanden Bos, T., Gimpel, S.D., Din, , Grabstein, K.H., Widmar, M.B., Park, L.S., Cosmann, D. & Beckmann, M.P. (1990) Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. *The Journal of Experimental Medicine*, 171, 861-873.

Itoh, N., Tsujimoto, Y. & Nagata, S. (1993) Effect of bcl-2 on Fas antigen-mediated cell death. *The Journal of Immunology*, 151, 621-627.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S-I., Sameshima, M., Hase, A., Seto, Y. & Nagata, S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediated apoptosis. *Cell*, 66, 233-243.

Jaattela, M., Wissing, D., Bauer, P.A. & Li, G.C. (1992) Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *The EMBO Jounrnal*, 11, 3507-3512.

Janssen, Y.M.W., Van Houten, B., Borm, P.J.A. & Mossman, B.T. (1993) Biology of disease: cell and tissue responses to oxidative damage. *Laboratory Investigation*, 69, 261-274.

Janssens, S.P., Shimouchi, A., Quertermous, T., Bloch, D.B. & Bloch, K.D. (1992) Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. *The Journal of Biological Chemistry*, 267, 14519-14522.

Jarvis, W.D., Kolesnick, R.N., Fornari, F.A., Traylor, R.S., Gewirtz, D.A. & Grant, S. (1994) Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. *Proceedings of the National Academy of Sciences, USA*, 91, 73-77.

Jenkins, M.K., Taylor, P.S., Norton, S.D. & Urfahl, K.B. (1991) CD28 delivers a costimulatory signal involved in antigen specific IL-2 production by human T cells. *The Journal of Immunology*, 147, 2461-2466.

Jokhi, P.P., Chumbley, G., King, A., Gardner, L. & Loke, Y.W. (1993) Expression of the colony stimulating factor-1 receptor (c-fms product) by cells at the human uteroplacental interface. *Laboratory Investigation*, 68, 308-320.

Kameda, T., Matsuzaki, N., Sawai, K., Okada, T., Saji, F.,Matsuda, T., Hirano, T., Kishimoto, T.& Tanizawa, O. (1990) Production of interleukin-6 by normal human trophoblast. *Placenta*, 11, 205-213.

Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. & Bredesen, D.E. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, 262, 1274-1276.

Kanzaki, H., Crainie, M., Lin, H., Yui, J., Guilbert, L.J., Mori, T. & Wegmann, T.G. (1991) The in situ expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA at the maternal-fetal interface. *Growth Factors*, 5, 69-74.

Kanzaki, H., Yui, J., Iwai, M., Imai, K., Kariya, M., Hatayama, H., Mori, T., Guilbert, L.J. & Wegmann, T.G. (1992) The expression and localization of mRNA for colony-stimulating factor (CSF-1) in human term placenta. *Human Reproduction*, 7, 563-567.

Kao, L.C., Caltabiano, S., Wu, S., Strauss, J.F. & Kliman, H.J. (1988) The human villous cytotrophoblast: interactions with extracellular matrix proteins, endocrine function and cytoplasmic differentiation in the absence of syncytium formation. *Developmental Biology*, 130, 693-702.

Karasaki, Y., Jaken, S., Komoriya, A. & Zoon, K.C. (1989) Phorbol ester and interferon-gamma modulation of epidermal growth factor receptors on human amniotic (WISH) cells. *The Journal of Biological Chemistry*, 264, 6158-6163.

Karl, P.I., Alpy, K.L. & Fisher, S.E. (1992) Serial enzymeatic digestion method for isolation of human placental trophoblasts. *Placenta*, 13, 385-387.

Kaufmann, P., Stark, J.& Stegner, H.E. (1977) The villous stroma of the human placenta. The ultrastucture of fixed connective tissue ce s. *Cell and Tissue Research*, 177, 105-121.

Kauma, S.W. (1993) Interleukin-1β stimulates colony-stimulating factor-1 production in human term placenta. *Journal of Clinical Endocrinology and Metabolism*, 76, 701-703.

Kauma, S.W., Turner, T.T. & Harty, J.R. (1994) Interleukin-1β stimulates interleukin-6 production in placental villous core mesenchymal cells. *Endocrinology*, 134, 457-460.

Kauma, S.W., Aukerman, S.L., Eierman, D., [illegible] T. (1991) Colony-stimulating factor-1 and *c-fms* expression in [illegible]metrial tissues and placenta during the menstrual cycle and [illegible]gnancy. *Journal of Clinical Endocrinology and Metabolism*, 73, 746-751.

Kauma, S.W., Walsh, S.W., Nestler, J.E. & Turner, T.T. (1992) Interleukin-1 is induced in the human placenta by endotoxin and isolation procedures for trophoblasts. *Journal of Clinical Endocrinology and Metabolism*, 75, 951-955.

Kauma, S.W., Herman, K., Wang, Y. & Walsh, S.W. (1993) Differential mRNA expression and production of interleukin-6 in placental trophoblast and villous core compartments. *American Journal of Reproductive Immun[illegible]*, 30, 131-135.

Kesson, [illegible]arwich, R.F., Kazazi, F., Mathijs, J.M., Chang, J., King, N.J.C.[illegible]ingham, A.L. (1993) Human immunodeficiency virus type I infection of human placen[illegible]acrophages in vitro. *The Journal of Infectious Disease*, 168, 571-579.

Kim, M-Y., Linardic, C., Obeid, L. & Hannun, Y. (1991) Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor α and γ-interferon. *The Journal of Biological Chemistry*, 266, 484-489.

Kishimoto, T. & Hirano, T. (1988) Molecular regulation of B-lymphocyte response. *Annual Review of Immunology*, 6, 485-512.

Kitamura, T., Sato, N., Arai, K. & Miyajima, A. (1991) Expression cloning of the human IL-3 receptor cDNA reveals [illegible]red β subunit for the human IL-3 and GM-CSF receptors. *Cell*, 66, 116[illegible]4.

Kliman, H.J., J.E. Nestler, E. Sermasi, J.M. Sanger & J.F. Strauss III. 1986. Purification, characterization and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology*, 118, 1567-1582.

Kliman, H.J., Coutifaris, C., Babalola, G.O., Soto, E.A., Kao, L-C., Queenan, Jr., J.T., Feinberg, R.F. & Strauss III, J.F. (1989) The human trophoblast: homotypic and heterotypic cell-cell interactions. In *Development of Preimplantation Embryos and Their Environment* (Ed.) Yoshinaga, K. & Mori, T. pp.425-434. New York: Alan R. Liss Inc.

Kohno, T., Brewer, B.T., Baker, S.L., Schwartz, P.E., King, M.W., Hale, K.K., Squires, C.H., Thompson, R.C. & Vannice, J.L. (1990) A second tumor necrosis factor receptor gene product can shed a naturally occurring tumor necrosis factor inhibitor. *Proceedings of the National Academy of Sciences, USA*, 87, 8331-8335.

Kolesnick, R. & Golde, D.W. (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell*, 77, 325-328.

Kolb, H. & Kolb-Bachofen, V. (1992) Nitric oxide: a pathogenetic factor in autoimmunity. *Immunology Today*, 13, 157-164.

Korhonen, M., Ylanne, J., Laitinen, L., Cooper, H.M., Quaranta, V. & Virtanen, I. (1991) Distribution of the α1-α6 integrin subunits in human developing and term placenta. *Laboratory Investigation*, 65, 347-356.

Korsmeyer, S.J. (1992) Bcl-2: a repressor of lymphocyte death. *Immunology Today*, 13, 285-288.

Kovats, S., Main, E.K., Librach, C., Stubblebine, M., Fisher, S.J. & Demars, R. (1990) A class I antigen, HLA-G, expressed in human trophoblasts. *Science*, 248, 220-223.

Kriegler, M., Perez, C., DeFay, K., Albert, I. & Lu, S.D. (1988) A novel form of TNF/cachetin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell*, 53, 45-53.

Kumar, S. & Baglioni, C. (1991) Protection from tumor necrosis factor-mediated cytolysis by overexpression of plasminogen activator inhibitor type-2. *The Journal of Biological Chemistry*, 266, 20960-20964.

Kunkel, S.L., Standiford, T., Kasahara, K. & Strieter, R.M. (1991) Interleukin-8 (IL-8) : the major neutrophil chemotactic factor in the lung. *Experimental Lung Research*, 17, 17-23.

Ladner, M.B., Martin, G.A., Noble, J.A., Nikoloff, D.M., Tal, T., Kawasaki, E.S. & White, T.J. (1987) Human CSF-1: gene structure and alternative splicing of mRNA precursors. *EMBO Journal*, 6, 2693-2698.

Lambert, L.E., Whitten, J.P., Baron, B.M., Cheng, H.C., Doherty, N.S. & McDonald, I.A. (1991) Nitric oxide synthesis in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sciences*, 48, 69-75.

Lairmore, M.D., Cuthbert, PS., Utley, L.L., Morgan, C.J., Dezzutti, C.S., Anderson, C.L. & Sedmak, D.D. (1993) Cellular localization of CD4 in the human placenta. Implications for maternal-to-fetal transmission of HIV. *The Journal of Immunology*, 151, 1673-1681.

Langer, J.A. and Pestka, S. (1988) Interferon receptors. *Immunology Today*, 9, 393-400.

Larrick, J.W. & Wright, S.C. (1990) Cytotoxic mechanism of tumor necrosis factor-α. *FASEB Journal*, 4, 3215-3223.

Laster, S.M., Wood, J.G. & Gooding, L.R. (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. The *Journal of Immunology*, 141, 2629-2634.

Lea, R.G., Flanders, K.C., Harley, C.B., Manuel, J., Banwatt, D. & Clark, D.A. (1992) Release of a transforming growth factor (TGF)-β2-related suppressor factor from postimplantation murine decidual tissue can be correlated with the detection of a subpopulation of cells containing RNA for TGF-β2. *The Journal of Immunology*, 148, 778-787.

LeBrun, D.P., Warnke, R.A. & Cleary, M.L. (1993) Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. *American Journal of Pathology*, 142, 743-753.

Lewis, S.H., Reynolds-Kohler, C., Fox, H.E. & Nelson, J.A. (1990) HIV-1 in trophoblastic and villous Hofbauer cells, and haematological precursors in eight-week fetuses. *The Lancet*, 335, 565-568.

Li, Y., Matsuzaki, N., Masuhiro, K., Kameda, T., Taniguchi, T., Saji, F., Yone, K. & Tanizawa, O. (1992) Trophoblast-derived tumor necrosis factor-α induces release of human chorionic gonadotropin using interleukin-6 (IL-6) and IL-6-receptor-dependent system in the normal human trophoblasts. *Journal of Clinical Endocrinology and Metabolism*, 74, 184-191.

Lifson, J.D., Reyes, G.R., Mcgrath, M.S., Stein, B.S. & Engleman, E.G. (1986) AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. *Science*, 232, 1123-1127.

Lin, J.Y. and Chadee, K. (1992) Macrophage cytotoxicity against *Enamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. *The Journal of Immunology*, 148, 3999- 4005.

Lin, H., Mosmann, T.R., Guilbert, L., Tuntipopipat, S. & Wegmann, T.G. (1993) Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *The Journal of Immunology*, 151, 4562-4573.

Loeffler, C.M., Smyth, M.J., Longo, D.L., Kopp, W.C., Harvey, L.K., Tribble, H.R., Tase, J.E., Urba, W.J., Leonard, A.S., Young, H.A. & Ocha, A.C. (1992) Immunoregulation in cancer-bearing hosts: downregulation of gene expression and cytotoxic function in $CD8^+$ T cells. *The Journal of Immunology*, 149, 949-956.

Loetscher, H., Pan, Y-C E., Lahm, H-W., Gentz, R., Brockhaus, M., Tabuchi, H. & Lesslauer, W. (1990) Molecular cloning and expression of the human 55kd tumor necrosis factor receptor. *Cell*, 61, 351-359.

Loetscher, H., Steuber, D., Banner, D., Mackay, F. & Lesslauer, W. (1993) Human tumor necrosis factor α (TNFα) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors. *The Journal of Biological Chemistry*, 268, 26350-26357.

Loke, Y.W. & Butterworth, B.H. (1987) Heterogeneity of human trophoblast populations. In *Immunoregulation and Fetal Survival* (Ed.) Gill III, T.J.,

Wegmann, T.G. & Nisbet-Brown, E. pp. 197-209. New York: Oxford Univeristy Press.

Loke, Y.W. & King, A. (1990) Current topic: interferon and human placental development. *Placenta*, 11, 291-299.

Loke, Y.W., Butterworth, B.H., Margetts, J.J. & Burland, K. (1986) Identification of cytotrophoblast colonies in cultures of human placental cells using monoclonal antibodies. *Placenta*, 7, 221-231.

Loke, Y.W., King, A., Gardner, L. & Carter, N.P. (1992) Evidence for the expression of granulocyte-macrophage colony-stimulating factor receptors by human first trimester extravillous trophoblast and its response to this cytokine. *Journal of Reproductive Immunology*, 22, 33-45.

Lowenstein, C.J., Glatt, C.S., Bredt, D.S.& Snyder, S.H. (1992) Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proceedings of the National Academy of Sciences, USA*, 89, 6711-6715.

Luger, T.A. & Schwarz, T. (1990) Evidence for an epidermal cytokine network. *Journal of Investigative Dermatology*, 95, 100S-104S.

Malorni, W., Rivabene, R., Santini, M.T. & Donelli, G. (1993) N-acetylcysteine inhibits apoptosis and decreases viral particles in HIV-chronically infected U937 cells. *FEBS*, 327, 75-78.

Mangan, D.F. & Wahl, S.M. (1991) Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. *The Journal of Immunology*, 147, 3408-3412.

Mano, H. and Chermann, J.C. (1991) Replication of human immunodeficiency virus type 1 in primary cultured placental cells. *Research in Virology*, 142, 95-104.

Martin, B.J. & Spicer, S.S. (1973) Ultrastructural features of cellular maturation and aging in human trophoblast. *Journal of Ultrastructural Research*, 43, 133-149.

Maruo, T., Matsuo, H., Oishi, T., Hayashi, M., Nishino, R. & Mochizuki, M. (1987) Induction of differentiated trophoblast function by epidermal

growth factor: relation of immunohistochemically detected cellular epidermal growth factor receptor levels. *Journal of Clinical Endocrinology and Metabolism*, 64, 744-750.

Marsden, P.A., Schappert, K.T., Chen, H.S., Flowers, M., Sundell, C.L., Wilcox, J.N., Lamas, S.& Michel, T. (1992) Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS*, 307, 287-293.

Masuhiro, K., Matsuzake, N., Nishino, E., Taniguchi, T., Kameda, T., Li, Y., Saji, F. & Tanizawa, O. (1991) Trophoblast-derived interleukin-1 (IL-1) stimulates the release of human chorionic gonadotropin by activating IL-6 and IL-6 receptor system in first trimester human trophoblasts. *Journal of Clinical Endocrinology and Metabolism*, 72, 594-601.

Matthews, N., Neale, M.L., Jackson, S.K. & Stark, J.M. (1987) Tumor cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology*, 62, 153-155.

Meade, R., Askenase, P.W., Geba, G.P., Neddermann, K., Jacoby, R.O. & Pasternak, R.D. (1992) Transforming growth factor-β1 inhibits murine immediate and delayed type hypersensitivity. *The Journal of Imn.unology*, 149, 521-528.

Meltzer, M.S., Skillman, D.R., Hoover, D.L., Hanson, B.D., Turpin, J.A., Kalter, C.D. & Gendelman, H.E. (1990) Macrophages and the human immunodeficiency virus. *Immunology Today*, 11, 217-223.

Merrill, J.E., Ignarro, L.J., Sherman, M.P., Melinek, J. & Lane, T.E. (1993) Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *The Journal of Immunology*, 151, 2132-2141.

Metcalf, D. (1991) Control of granulocytes and macrophages: molecular, cellular, and clinical aspects. *Science*, 254, 529-533.

Metcalf, D., Nicola, N.A., Gearing, D. & Gough, N.M. (1990) Low-affinity placenta-derived receptors for human granulocyte macrophage colony-stimulating factor can deliver a proliferative signal to murine hemopoietic cells. *Proceedings of the National Academy of Sciences, USA*, 87, 4670-4674.

Miller, D.A., Pelton, R.W., Derynck, R. & Moses, H.L. (1990) Transforming growth factor-β: A family of growth regulatory peptides. *Annals of the New York Academy of Sciences*, 593, 208-217.

Mitchell. M.D., Trautman, M.S. & Dudley, D.J. (1993) Cytokine networking in the placenta. *Placenta*, 14, 249-275.

Moore, K.W., Vieira, P., Fiorentino, D.F., Trounstine, M.L., Khan, T.A. & Mosmann, T.R. (1990) Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRF1. *Science*, 248, 1230-1234.

Morrish, D.W., Marusyk, H. & Siy, O. (1987) Demonstration of specific secretory granules for human chorionic gonadotropin in placenta. *The Journal of Histochemistry and Cytochemistry*, 35, 93-101.

Morrish, D.W., Marusyk, H. & Bhardwaj, D. (1988) Ultrastructural localization of human placental lactogen in distinctive granules in human term placenta : comparison with granules containing human chorionic gonadotropin. *The Journal of Histochemistry and Cytochemistry*, 36, 193-197.

Morrish, D.W., Bhardwaj, D. & Paras, M.T. (1991) Transforming growth factor β1 inhibits placental differentiation and human chorionic gonadotropin and human placental lactogen secretion. *Endocrinology*, 129, 22-26.

Morrish, D.W., Bhardwaj, D., Dabbagh, L.K., Marusyk, H. & Siy, O. (1987) Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta, *Journal of Clinical Endocrinology and Metabolism* 65, 1282-1290.

Morrish, D.W., Shaw, A.R.E., Seehafer, J., Bhardwaj, D. & Paras, M.T. (1991) Preparation of fibroblast-free cytotrophoblast cultures utilizing differential expression of the CD9 antigen. *In Vitro Cellular and Developmental Biology*, 27A: 303-306.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, 55-63.

Mosmann, T.R. & Moore, K.W. (1991) The role of IL-10 in crossregulation of T_H1 and T_H2 reponses. *Immunology Today*, 49-53.

Mosmann, T.R. Properties and functions of interleukin 10. *Advances in Immunology*. (in press)

Mohler, K.M., Torrance, D.S., Smith, C.A., Goodwin, R.G., Stremler, K.E., Fung, V.P., Madani, H. & Widmer, M.B. 1993 Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. *The Journal of Immunology*, 151, 1548-1561.

Nakano, T., Ohara, O., Teraoka, H. & Arita, H. (1990) Glucocorticoids suppress group II phospholipase A_2 production by blocking RNA synthesis and post-transcriptional expression. *The Journal of Biological Chemistry*, 265, 12745-12749.

Nishino, E., Matsuzaki, N., Masuhiro, K., Kameda, T., Taniguchi, T., Takagi, T., Saji, F. & Tanizawa, O. (1990) Trophoblast-derived interleukin-6 (IL-6) regulates human chorionic gonadotropin release through IL-6 receptor on human trophoblasts. *Journal of Clinical Endocrinology and Metabolism*, 71, 436-441.

Novick, D., Cohen, B. & Rubinstein, M. (1994) The human interferon α/β receptor: characterization and molecular cloning. *Cell*, 77, 391-400.

Obeid, L.M., Linardic, C.M., Karolak, L.A. & Hannun, Y.A. (1993). Programmed cell death induced by ceramide. *Science*, 259, 1769-1771.

Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R. & Sikorska, M. (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO Journal*, 12, 3679-3684.

Ohashi, K., Saji, F., Kato, M., Wakimoto, A. & Tanizawa O. (1992) Tumor necrosis factor-α inhibits human chorionic gonadotropin secretion. *Journal of Clinical Endocrinology and Metabolism*, 74, 130-134.

Ohlsson, R. (1989) Growth factors, protooncogenes and human placental development. *Cell Differentiation and Development*, 28, 1-16.

Old, L.J. (1985) Tumor necrosis factor. *Science*, 230, 630-632.

Opipari, Jr, A.W., Hu, H.M., Yabkowitz, R. & Dixit, V.M. (1992) The A20 zinc finger protein protects cells from tumor necrosis factor cytotoxicity. *The Journal of Biological Chemistry*, 267, 12424-12427.

Owen-Schaub, L.B., Yonehara, S., Crump III, W.L. & Grimm, E.A. (1992) DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. *Cellular Immunology*, 140, 197-205.

Palladino, M.A., Morris, R.E., Fletcher Starnes, H.& Levinson, A.D. (1990) The transforming growth factor-betas: a new family of immunoregulatory molecules. *Annals of the New York Academy of Sciences*, 593, 181-187.

Pampfer, S. Tabibzaden, S., Chuan, F-C. & Pollard, J.W. (1991) Expression of colony-stimuting factor-1 (CSF-1) messenger RNA in human endometrial glands during the menstrual cycle: molecular cloning of a novel transcript that predicts a cell surface form of CSF-1. *Molecular Endocrinology*, 5, 1931-1938.

Pampfer, S., Daiter, E., Barad, D., & Pollard, J.W. (1992) Expression of the colony-stimulating factor-1 receptor (*c-fms* proto-oncogene product) in the human uterus and placenta. *Biology of Reproduction*, 46, 48-57.

Pantazis, P., Kharbanda, S., Goustin, A.S., Galanopoulos, T. & Kufe, D. (1991) Coexpression of the genes for platelet-derived growth factor B-chain receptor and macrophage colony-stimulating factor 1 receptor during monocytic differentiation. *Proceedings of the National Academy of Sciences, USA*, 88, 2481-2485.

Pasqualini, J.R. & Kincl, F. (1985) Hormones and the Fetus (Volume I). pp 173-334. Oxford: Pergamon Press.

Peyman, J.A. & Hammond, G.L. (1992) Localization of IFN-γ receptor in first trimester placenta to trophoblasts but lack of stimulation of HLA-DRA,

-DRB, or invariant chain mRNA expression by IFN-γ. *The Journal of Immunology*, 149, 2675-2680.

Peyman, J.A., Nelson, P.J. & Hammond, G.L. (1992) HLA-DR genes are silenced in human trophoblasts and stimulation of signal transduction pathways does not circumvent interferon-γ unresponsiveness. *Transplantation Proceedings*, 24, 470-471.

Pfeffer, K., Matsuyama, T., Kundig, T., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Kronke, M. & Mak, T.W. (1993) Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell*, 73, 457-467.

Pollard, J.W., Bartocci, A., Arceci, R., Orlofsky, A., Ladner, M.B. & Stanley, E.R. (1987) Apparent role of the macrophage growth factor, CSF-1, in placental development. *Nature*, 330, 484-486.

Pollard, J.W., Hunt, J.S., Wiktor-Jedrzejcak, W. & Stanley, E.R. (1991) A pregnancy defect in the osteopetrotic (*op/op*) mouse demonstrates the requirement for CSF-1 in female fertiliy. *Developmental Biology*, 148, 273-283.

Pruzanski, W. & Vadas, P. (1991) Phospholipase A2- a mediator between proximal and distal effectors of inflammation. *Immunology Today*, 12, 143-146.

Rabinovitch, A., Sumoski, W., Rajotte, R.V. & Warnock, G.L. (1990) Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. *Journal of Endocrinology and Metabolism*, 71, 152-156.

Rabinovitch, A., Saurez, W.L., Thomas, P.D., Strynadka, K. and Simpson, I. (1992) Cytotoxic effects of cytokines on rat islets: evidence for involvement of free radicals and lipid peroxidation. *Diabetologia*, 35, 409-413.

Ranges, G.E., Figari, I.S., Espevik, T. & Palladino, Jr., M.A. (1987) Inhibition of cytotoxic T cell development by transforming growth factor β and reversal by recombinant tumor necrosis factor α. *The Journal of Experimental Medicine*, 166, 991-998.

Reid, T.R., Torti, F.M. & Ringold, G.M. (1989) Evidence for two mechanisms by which tumor necrosis factor kills cells. *The Journal of Biological Chemistry*, 264, 4583-4589.

Rettenmier, C.W. & Roussel, M.F. (1988) Differential processing of colony-stimulating factor 1 precursors encoded by two human cDNAs. *Molecular and Cellular Biology*, 8, 5026-5034.

Rettenmier, C.W., Sacca, R., Furman, W.L., Roussel, M.F., Holt, J.T., Nienhuis, A.W., Stanley, E.R. & Sherr, C.J. (1986) Expression of the human c-fms proto-oncogene product (colony-stimulating factor-1 receptor) on peripheral blood mononuclear cells and choriocarcinoma cell lines. *Journal of Clinical Investigation*, 77, 1740-1746.

Ringler, G.E., Coutifaris, C., Strauss III J.F., Allen, J.I. & Geier, M. (1989) Accumulation of colony-stimulating factor 1 in amniotic fluid during human pregnancy. *American Journal of Obstetrics and Gynecology*, 160, 655-666.

Roberts, W.M., Shapiro, L.H., Ashmun, R.A. & Look, A.T. (1992) Transcription of the human colony-stimulating factor-1 receptor gene is regulated by separate tissue-specific promoters. *Blood*, 79, 586-593.

Robertson, S.A., Mayrhofer, G. & Seamark, R.F. (1992) Uterine epithelial cells synthesize granulocyte-macrophage colony-stimulating factor and interleukin-6 in pregnant and nonpregnant mice. *Biology of Reproduction*, 46, 1069-1079.

Romero, R., Manogue, K.R., Mitchell, M.D., Wu, Y.K., Oyarzun, E., Hobbins, J.C. & Cerami, A. (1989) Cachetin-tumor necrosis factor in the amniotic fluid of women with intraamniotic infection and preterm labor. *American Journal of Obstetrics and Gynecology*, 161, 336-341.

Rook, G.A.W., Hernandez-Pando, R. & Lightman, S.L. (1994) Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. *Immunology Today*, 15, 301-303.

Rook, A.H., Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Sporn, M.B., Burlington, D.B., Lane, H.C. & Fauci, A.S. (1986) Effects of transforming growth factor β on the functions of natural killer cells:

depressed cytolytic activity and blunting of interferon responsiveness. *The Journal of Immunology*, 136, 3916-3920.

Rosenkranz, A.R., Schmaldienst, S., Stuhlmeier, K.M., Chen, W., Knapp, W. & Zlabinger, G.J. (1992) A microplate assay for the detection of oxidative products using 2',7'-dichlorofluorescin-diacetate. *Journal of Immunological Methods*, 156, 39-45.

Rossi, P. & Moschese, V. (1991) Mother-to-child transmission of human immunodeficiency virus. *FASEB Journal*, 5, 2419-2426.

Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M. & Bluethmann, H. (1993) Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature*, 364, 798-802.

Ruggiero, V., Tavernier, J., Fiers, W. & Baglioni, C. (1986) Induction of the synthesis of tumor necrosis factor receptors by interferon-γ. *The Journal of Immunology*, 136, 2445-2450.

Saito, S., Saito, M., Motoyoshi, K. & Ichijo, M. (1991) Enhancing effects of human macrophage colony-stimulating factor on the secretion of human chorionic gonadotropin by human chorionic villous cells and tPA30-1 cells. *Biochemical and Biophysical Research Communications*, 178, 1099-1104.

Schall, T.J., Lewis, M., Koller, K.J., Lee, A., Rice, G.C., Wong, G.H.W., Gatanaga, T., Granger, G.A., Lentz, R., Raab, H., Kohr, W.J., Goeddel, D.V. (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell*, 61, 361-370.

Schulze-Osthoff, K., Bakkers, A.C., Vanhaesebroeck, B., Beyaert, R., Jacob, W.W.A. & Fiers, W. (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions : evidence for the involvement of mitochondrial radical generation. *The Journal of Biological Chemistry*, 267, 5317-5322.

Selmaj, K., Raine, C.S., Farooq, M., Norton, W.T. & Brosnan, C.F. (1991) Cytokine cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin. *The Journal of Immunology*, 147, 1522-1529.

Shalaby, M.R., Sundan, A., Loetscher, H., Brockhaus, M., Lesslauer, W. & Espevik, T. (1990) Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. *Journal of Experimental Medicine*, 172, 1517-1520.

Sherr, C.J. (1990) Colony-stimulating factor-1 receptor. *Blood*, 75, 1-12.

Shi, Y., Szalay, M.G., Paskar, L., Boyer, M., Singh, B. & Green, D.R. (1992) Activation-induced cell death in T cell hybridomas is due to apoptosis: morphologic aspects and DNA fragmentation. *The Journal of Immunology*, 144, 3326-3333.

Silen, M.L., Firpo, A., Morgello, S., Lowry, S.F. & Francus, T. (1989) Interleukin-1α and tumor necrosis factor α cause placental injury in the rat. *American Journal of Pathology*, 135, 239-244.

Simon, C., Frances, A., Piquette, G., Hendrickson, M., Milki, A. & Polan, M.L. (1994) Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/ paracrine function. *Journal of Clinical Endocrinology and Metabolism*, 78, 847-854.

Smith, C.A., Farrah, T. & Goodwin, R.G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell*, 76, 959-962.

Smith, C.A., Davis, T., Anderson, D., Solam,, L., Beckmann, M.P., Jerzy, E., Dower, S.K., Cosman, D. & Goodwin, R.G. (1990) A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science*, 248, 1019-1023.

Sooranna, S.R. & Contractor, S.F. (1991) Vectorial transcytosis of immunoglobulin G by human term trophoblast cells in culture. *Experimental Cell Research*, 192, 141-145.

Soubiran, P., Zapitelli, J-P. & Schaffar, L. (1987) IL2-like material is present in human placenta and amnion. *Journal of Reproductive Immunology*, 12, 225-234.

Spies, T., Morton, C.C., Nedospasov, S.A., Fiers, W., Pious, D. & Strominger, J.L. (1986) Genes for the tumor necrosis factors α and β are

linked to the human major histocompatibility complex. *Proceedings of the National Academy of Sciences, USA*, 83, 8699-8702.

St. Louis, M.E., Kamenga, M., Brown, C., Nelson, A.M., Manzila, T., Batter, V., Behets, F., Kabagabo, U., Ryder, R.W., Oxtoby, M., Quinn, T.C. & Heyward, W.L. (1993) Risk for perinatal HIV-1 transmission according to maternal immunologic, virologic and placental factors. *JAMA*, 269, 2853-2859.

Stamler, S.S., Singel, D.J. & Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science*, 258, 1898-1901.

Stanley, E.R., Guilbert, L.J., Tushinski, R.J. & Bartelmez, S.H. (1983) CSF-1 - a mononuclear phagocyte lineage-specific hemopoietic growth factor. *Journal of Cellular Biochemistry*, 21, 151-159.

Starkey, P.M. (1991) Expression on cells of early human pregnancy decidua, of the p75, IL-2 and p145, IL-4 receptor proteins. *Immunology*, 73, 64-70.

Suffys, P., Beyaert, R., Van Roy, F. & Fiers, W. (1987) Reduced tumor necrosis factor-induced cytotoxicity by inhibitors of the arachidonic acid metabolism. *Biochemical and Biophysical Research Communications*, 149, 735-743.

Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, L.S., Palladino, M.A & Shepard, H.M. (1985) Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. *Science*, 230, 943-945.

Sumoski, W., Baquerizo, H. & Rabinovitch, A. (1989) Oxygen free radical scavengers protect rat islet cells from damage by cytokines. *Diabetologia*, 32, 792-796.

Stamler, J.S., Singel, D.J. & Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science*, 258, 1898-1901.

Strickland, S. and Richards, W.G. (1992) Invasion of the trophoblasts. *Cell*, 71, 355-357.

Stuehr, D.J. & Nathan, C.F. (1989) Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *Journal of Experimental medicine*, 169, 1543-1555.

Swat, W., Ignatowicz, L. & Kisielow, P. (1991) Detection of apoptosis of immature $CD4^{+}8^{+}$ thymocytes by flow cytometry. *Journal of Immunological Methods*, 137, 79-87.

Taga, K. & Tosato, G. (1992) IL-10 inhibits human T cell proliferation and IL-2 production. *The Journal of Immunology*, 148, 1143-1148.

Tangri, S. & Ragupathy, R. (1993) Expression of cytokines in placentas of mice undergoing immunologically mediated spontaneous fetal resorptions. *Biology of Reproduction*, 49, 850-856.

Tartaglia, L.A. & Goeddel, D.V. (1992) Two TNF receptors. *Immunology Today*, 13, 151-153.

Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino, M.A. & Goeddel, D.V. (1991) The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proceedings of the National Academy of Sciences, USA*, 88, 9292-9296.

Tartaglia, L.A., Ayres, T.M., Wong, G.H.W. & Goeddel, D.V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell*, 74, 845-853.

Tavernier, J., Devos, R., Cornelius, S., Tuypens, T., Van der Heyden, J., Fiers, W. & Paletinck, G. (1991) A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific α chain and a β chain shared with the receptor for GM-CSF. *Cell*, 66, 1175-1184.

Thoma, B., Grell, M., Pfizenmaier, K. & Scheurich, P. (1990) Identification of a 60-kD tumor necrosis factor (TNF) receptor as the major signal transducing component in TNF responses. *Journal of Experimental Medicine*, 172, 1019-1023.

Torti, F.M., Dieckmann, B., Beutler, B., Cerami, A.& Ringold, G.M. (1985) A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. *Science*, 229, 867-869.

Unnikumar, K.R., Wegmann, R. & Panigel, M. (1988) Immunohistochemical profile of the human placenta: studies on localization of prolactin, human chorionic gonadotropin, human placental lactogen, renin amd oxytocin. *Cellular and Molecular Biology*, 34, 697-710.

Valente, G., Ozmen, L., Novelli, F., Geuna, M., Palestro, G., Forni, G. & Garotta, G. (1992) Distribution of interferon-γ receptor in human tissues. *European Journal of Immunology*, 22, 2403-2412.

Vieira, P., de Waal Malefyt, R., Dang, M-N., Johnson, K.E., Kastelein, R., Fiorentino, D.F., deVries, J.E., Roncarolo, M-G., Mosmann, T.R. & Moore, K.W. (1991) Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: Homology to Epstein-Barr virus open reading frame BCRF1. *Proceedings of the National Academy of Sciences, USA*, 88, 1172-1176.

Vince, G., Shorter, S., Starkey, P., Humphreys, J., Clover, L., Wilkins, T., Sargent, I. & Redman, C. (1992) Localization of tumor necrosis factor production in cells at the materno/ fetal interface in human pregnancy. *Clinical and Experimental Immunology*, 88, 174-180.

Visvader, J. & Verma, I.M. (1989) Differential transcription of exon 1 of the human c-fms gene in placental trophoblasts and monocytes. *Molecular and Cellular Biology*, 9, 1336-1341.

Vuckovic, M., Genbacev, O. & Kumar, S. (1992) Immunohistochemical localization of transforming growth factor-beta in first and third trimester. *Pathobiology*, 60, 149-151.

Wahl, S.M., McCartney-Francis, N., Allen, J.B., Dougherty, E.B. & Dougherty, S.F. (1990) Macrophage production of TGF-β and regulation by TGF-β. *Annals of the New York Academy of Sciences*, 593, 188-196.

Walker, N.I., Bennett, R.E. & Kerr, J.F.R. (1989) Cell death by apoptosis during involution of the lactating breast in mice and rats. *The American Journal of Anatomy*, 185, 19-32.

Wegmann, T.G. (1990) The cytokine basis for cross-talk between the maternal immune and reproductive systems. *Current Opinion in Immunology*, 2, 765-769.

Wegmann, T.G., Athanassakis, I., Guilbert, L., Branch, D., Dy, M., Menu, E. & Chaouat, G. (1989) The role of M-CSF and GM-CSF in fostering placenta growth, fetal growth, and fetal survival. *Transplantation Proceedings*, 21, 566-568.

Wegmann, T.G., Lin, H., Guilbert, L. & Mosmann, T.R. (1993) Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a T_H2 phenomenon? *Immunology Today*, 14, 353-356.

Wiegmann, K., Schutze, S., Kampen, E., Himmler, A., Machleidt, T. & Kronke, M. (1992) Human 55-kDa receptor for tumor necrosis factor coupled to signal transduction cascades. *The Journal of Biological Chemistry*, 267, 17997-18001.

Wiktor-Jedrzejcak, W., Bartocci, A., Ferrante, Jr., A.W., Ahmed-Ansari, A., Sell, K.W., Pollard, J.W. & Stanley, E.R. (1990) Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (*op/op*) mouse. *Proceedings of the National Academy of Sceince, USA*, 87, 4828-4832.

Wolvekamp, M.C.J. & Marquet, R.L. (1990) Interleukin-6: historical background, genetics and biological significance. *Immunology Letters*, 24, 1-10.

Wong, G.H.W., Elwell, J.H., Oberley, L.W.& Goeddel, D.V. (1989) Manganous superoxide dismutase is essential for cellular resistance to cytotoxictiy of tumor necrosis factor. *Cell*, 58, 923-931.

Wong, G.H.W., Tartaglia, L.A., Lee, M.S. & Goeddel, D.V. (1992) Antiviral activity of tumor necrosis factor (TNF) is signaled through the 55-kDa receptor, type I TNF. *The Journal of Immunology*, 149, 3350-3353.

Wood, G.W. (1980) Mononuclear phagocytes in the human placenta. *Placenta*, 1, 113-123.

Wright, C.D., Mulsch, A., Busse, R. & Osswald, H. (1989) Generation of nitric oxide by human neutrophils. *Biochemical and Biophysical Research Communications*, 160, 813-819.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J. & Mathison, J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, 249, 1431-1433.

Xie, Q., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T. & Nathan, C. (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*, 256, 225-228.

Yagel, S., Lala, P.K., Powell, W.A. & Casper, R.F. (1989) Interleukin-1 stimulates human chorionic gonadotropin secretion by first trimester human trophoblast. *Journal of Clinical Endocrinology and Metabolism*, 68, 992-995.

Yamauchi, N., Kuriyama, H., Watanabe, N., Neda, H., Maeda, M., Niitsu, Y. (1989) Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells in vitro. *Cancer Research*, 49, 1671-1675.

Yanaga, F. & Watson, S.P. (1992) Tumor necrosis factor a stimulates sphingomyelinase through the 55 kDa receptor in HL-60 cells. *FEBS*, 314, 297-300.

Yang, Y., Yelavarthi, K.K., Chen, H-L., Pace, J.L., Terranova, P.F. & Hunt, J.S. (1993) Molecular, biochemical, and functional characteristics of tumor necrosis factor-α produced by human placental cytotrophoblastic cells. The *Journal of Immunology*, 150, 5614-5624.

Yeger, H., Loines, L.D., Wong, P. & Silver, M.M. (1989) Enzymatic isolation of human trophoblast and culture on various substrates: comparison of first trimester with term trophoblast. *Placenta*, 10, 137-151.

Yelavarthi, K.K. & Hunt, J.S. (1993) Analysis of p60 and p80 tumor necrosis factor-α receptor messenger RNA and protein in human placentas. *American Journal of Pathology*, 143, 1131-1141.

Yoshida, H., Hayashi, S-I., Kunisada, T., Ogawa, M. Nishikawa, H., Sudo, T., Shultz, L.D. & Hishikawa, S-I. (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*, 345, 442-444.

Yssel, H., de Waal Malefyt, R., Roncarolo, M-G., Abrams, J.S., Lahesmaa, R., Spits, H. & de Vries, J.E. (1992) IL-10 is produced by subsets of human CD4+ T cell clones and peripheral blood T cells. *The Journal of Immunology*, 149, 2378-2384.

Yui, J., Garcia-Lloret, M., Brown, A.J., Berdan, R.C., Morrish, D.W., Wegmann, T.G. & Guilbert, L.J. (1994) Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. *Placenta*, 15, 231-246.

Zhou, Y., Damsky, C.H., Chiu, K., Roberts, J.M. & Fisher, S.J. (1993) Preeclampsia is associated with abnormal expression of adhesion molecules by invasive trophoblasts. *Journal of Clinical Investigation*, 91, 950-960.

Ziegler-Heitbrock, H.W.L. & Ulevitch, R.J. (1993) CD14: cell surface receptor and differentiation marker. *Immunology Today*, 14, 121-125.

Zimmerman, R.J., Marfino, B.J., Chan, A., Landre, P. & Winkelhake, J.L. (1989) The role of oxidant injury in tumor cell sensitivity to recombinant human tumor necrosis factor in vivo. *The Journal of Immunology*, 142, 1405-1409.

Zuckermann, F.A. & Head, J.R. (1986) Expression of MHC antigens on murine trophoblast and their modulation by interferon. *The Journal of Immunology*, 137, 846-853.