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Immune response on rabbit does of different genetic types subjected to reproductive, environment and immunologic challenges

DOCTORAL THESIS

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Y para que conste, firmo el presente en Valencia, a dieciocho de diciembre de dos mil doce.

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Dedicada a mi familia
y a mis amigos

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ABSTRACT

Rabbit health is considered a main handicap on commercial farms. The overall objective of this thesis was to study the immune response of rabbit does of different genetic types subjected to reproductive, environment and immunologic challenges, and its relationship with body condition. Effect of selection was studied using different genetic types. Three studies were carried out:

In the first study, the main objectives were to compare the peripheral blood lymphocyte populations of multiparous rabbit does under two reproductive rhythms [insemination at 11 days post partum (dpp) and weaning at 28 dpp; insemination at 25 dpp and weaning at 42 dpp], and to assess the influence on those of kits. Additionally, does' perirenal fat thickness was also measured at partum and weaning to observe if body condition correlated with lymphocyte populations. During whole lactation, counts of total $CD5^+$, $CD4^+$ and $CD8^+$ lymphocytes of females were generally lower in rabbit does weaned at 42 dpp as compared to 28 dpp. Moreover, counts of total B and $CD5^+$ lymphocytes in rabbit does weaned at 42 dpp correlated with their body condition, unlike that observed in rabbit does weaned at 28 dpp. Some correlations between lymphocyte counts in both groups of does and weaning rabbits were observed. At weaning, those young rabbits weaned at 42 dpp had a lower number of $CD4^+$ lymphocytes than those weaned at 28 dpp. In conclusion, the 42-ddp rabbit does presented a lower number of total lymphocytes and lymphocyte subsets during lactation and at weaning, and a poorer adaptation capacity during the gestation-lactation cycle.

In the second study, the effect of selection for reproduction (V line, selected for litter size at weaning) or foundation for reproductive longevity (the LP line) was investigated under normal and heat stress conditions. The cross between two maternal lines (V36 animals) was compared with the same cross, but 20 generations earlier, using a cryopreserved control population (V16 animals). To evaluate the possible correlation between energy balance and the immunological status of females, daily feed intake (DFI) during lactation week 2 and perirenal fat thickness (PFT) on 14 dpp were controlled by ultrasound. The results show that housing under heat stress conditions reduced the B lymphocytes counts in female rabbits. The highest lymphocytes population value in blood (total, T $CD5^+$, $CD4^+$ and $CD8^+$) was noted at first parturition, while the B lymphocytes count was lower at second parturition. Selection for litter size at weaning (V females) reduced the average counts of total and B lymphocytes in blood, mainly because these populations in V36 females continuously lowered from first to second parturition under normal housing conditions. Thus, more selected females (V36) at second parturition showed lower counts in blood for total T $CD5^+$ and $CD25^+$ lymphocytes. The main differences in blood counts between V36 and V16 females disappeared when housed under heat stress conditions, except for T $CD5^+$ and $CD25^+$, which increased in the V16 *vs.* V36 females on day 10 post-partum. Under normal conditions, no differences between LP and V36 females were found for most lymphocyte populations, only higher counts in $CD25^+$ for LP females. However, the lymphocytes counts (especially total and T $CD5^+$) of LP females increased under heat *vs.* normal conditions when lymphocytes populations obtained the lowest values (second parturition), while V36 females' counts remained invariable. Feed intake and females' perirenal fat thickness positively correlated with B lymphocytes counts in the blood of primiparous rabbit females in week 2 of lactation. In conclusion, these results indicate that selection for litter size at weaning might diminish animals' immune system response and adaptation capacity, while

the foundation for reproductive longevity criteria leads to more robust rabbit females as they present greater modulation under heat stress conditions when the immune system is affected.

The third study aimed to evaluate differences in the immune response in maternal lines of reproductive rabbit does, founded for the same criteria as in the previous study (V and LP lines). Females were subjected to three different reproductive effort levels: post-partum (PP) mating at first lactation and 9 kits at the second; post-weaning (PW) mating at first lactation and 9 kits at the second; and PW mating at first lactation and 5 kits during the second. At second weaning (30 dpp), an acute response was induced by an intravenous infusion of lipopolysaccharide (LPS). LP females seemed to be less affected during the hyperacute phase than V females, presenting lower plasma glucose content at 1.5 h post-infusion (pi) and rectal temperature at 6 h pi; they showed a greater ulterior immune response, with higher levels of c-reactive protein at 48 h pi and haptoglobin in plasma from 24 h pi. The survival test conferred a higher culling risk for V than for LP females during the first post-challenge hours. Moreover, the advantage of the LP in terms of survival rate over the V line came exclusively from the females mated after a first weaning (PW), which were also the main group responsible for the increase of NEFA in blood after 6 h pi. These results suggest that, with regard to immune response to LPS challenge, foundation by hyperlongevity productive criteria (LP females) contributes to obtain a more robust population of rabbit does characterized by improved response ability.

In conclusion, from the results obtained in the three thesis studies, it may conclude that there seems to be an evident relationship between body condition and health status of reproductive rabbit does as evident positive correlations were observed between body condition and main lymphocyte population counts in the first two studies, and non-esterified fatty acids (NEFAs) levels in blood seem to be the only plasma trait relating to female survival in the immunological challenge developed in the third study. Thus, these results confirm the initial thesis hypothesis which proposed that body condition is proposed to play a mediating role and entails that, for animals at the same production and genetic level, the better the body condition, the better the immune response.

RESUMEN

La salud de los animales puede ser considerada como uno de los principales obstáculos en las granjas cunícolas comerciales. El objetivo general de esta tesis doctoral, fue estudiar la respuesta inmune en conejas reproductoras de diferentes tipos genéticos sometidas a desafíos reproductivos, ambientales e inmunológicos y su relación con la condición corporal. Para ello se llevaron a cabo tres estudios.

En el primer estudio, los principales objetivos fueron comparar las poblaciones linfocitarias en sangre periférica de conejas multíparas bajo dos ritmos reproductivos: (1) inseminación artificial a los 11 días post-parto (dpp) con un destete a los 28 dpp con (2) inseminación a 25 dpp y destete a los 42 dpp]. También se estudió las poblaciones linfocitarias sanguíneas de los gazapos de las hembras anteriormente señaladas. Adicionalmente, se valoró el espesor de la grasa perirenal al parto y destete para observar si la condición corporal se correlacionaba con las poblaciones linfocitarias. Durante el ciclo de lactación, el recuento de linfocitos totales, $CD5^+$, $CD4^+$ y $CD8^+$ fueron generalmente más bajos en las conejas destetadas a los 42 dpp comparadas con las destetadas a 28 dpp. Además, los recuentos de linfocitos totales, linfocitos B y $CD5^+$ en conejas destetadas a los 42 dpp se correlacionaron con su condición corporal, contrariamente a lo observado en las hembras destetadas a los 28 dpp. También se observaron correlaciones entre los recuentos linfocitarios de los dos grupos de hembras y sus gazapos. De tal forma que en el momento del destete, los gazapos destetados a los 42 dpp presentaron un menor número de linfocitos $CD4^+$ que los destetados a los 28 dpp. En conclusión, las conejas destetadas a los 42 dpp presentaron un menor número de linfocitos totales y subpoblaciones de éstos durante la lactación y en el destete, así como menor capacidad de modulación de dichas poblaciones celulares durante el ciclo de gestación-lactación.

En el segundo trabajo se estudió el efecto de la selección por reproducción (línea V, seleccionada por tamaño de camada al destete, utilizando dos grupos de animales -V16 y V36- separados por 20 generaciones) o por longevidad reproductiva (línea LP), en condiciones normales y de estrés por calor. Para evaluar la posible correlación entre el balance energético y el estado inmunológico de las conejas, se controló el consumo diario de alimento (DFI) durante la segunda semana de la lactación y espesor de la grasa perirenal (PFT) el 14 dpp. Respecto a los resultados, se observó un menor recuento de linfocitos B en la cámara climática. Los recuentos más altos de linfocitos sanguíneos (totales, T $CD5^+$, $CD4^+$ y $CD8^+$) se observaron en el primer parto, mientras que los recuentos de linfocitos B fueron menores en el segundo parto. La selección por tamaño de camada al destete (hembras V) redujo los recuentos promedio de linfocitos totales y B en la sangre, principalmente porque estas poblaciones en las hembras V36 disminuyeron de manera continua desde el primer al segundo parto, en condiciones normales de alojamiento. Por lo tanto, las conejas de la línea V y de la generación actual (V36) mostraron en el segundo parto menores recuentos de linfocitos totales, T $CD5^+$ y $CD25^+$. Estas diferencias en los recuentos sanguíneos entre V36 y V16 desaparecieron cuando los animales fueron alojados bajo condiciones de estrés térmico, excepto para las poblaciones de linfocitos T $CD5^+$ y $CD25^+$, que aumentaron en las hembras V16en el día 10 post-parto. En condiciones normales, no hubo diferencias entre las hembras LP y V36 para la mayoría de las poblaciones linfocitarias, únicamente se observaron recuentos más elevados de linfocitos $CD25^+$ en las conejas LP. Sin embargo, los recuentos de linfocitos (especialmente totales y T $CD5^+$) de las hembras LP aumentaron en

cámara climática frente a la cámara convencional, mientras que los recuentos de las V36 permanecieron invariables. Se encontraron correlaciones positivas entre el consumo de alimento y el espesor de la grasa perirenal, con los recuentos de linfocitos B de las conejas en la segunda semana de lactación. Estos resultados indican que la selección por tamaño de camada al destete, podría haber tenido efectos perniciosos en el sistema inmunológico de los animales, de tal forma que muestran un menor número en algunas poblaciones linfocitarias y una peor respuesta inmune, mientras que la selección por criterios de híper-longevidad reproductiva habría conducido a la obtención de animales más robustos, ya que presentan mayores recuentos linfocitarios bajo condiciones de estrés térmico, cuando el sistema inmune se ve gravemente afectado.

En el tercer estudio se continuó evaluando las diferencias entre la respuesta inmune de distintas líneas maternas de conejas reproductoras empleando, para ello, hembras seleccionadas por los mismos criterios del estudio anterior. Las conejas reproductoras fueron sometidas a tres niveles diferentes de esfuerzo reproductivo: (1) conejas cubiertas post-parto (PP) en la primera semana de lactación y con 9 gazapos durante el segundo parto; (2) conejas cubiertas post-destete (PW) en la primera semana de lactación y con 9 gazapos durante el segundo parto y (3) conejas cubiertas post-destete (PW) en la primera semana de lactación y 5 gazapos durante el segundo parto. Al segundo destete (30 dpp), se indujo una respuesta inflamatoria aguda mediante la infusión intravenosa de lipopolisacárido de pared bacteriana (LPS). Las hembras LP parecieron estar menos afectadas durante la fase aguda que las hembras V, mostrando menores niveles de glucosa en plasma sanguíneo a las 1,5 horas post inoculación (pi) y una menor temperatura rectal a las 6 h pi, además mostraron una mayor respuesta inmunitaria ulterior, con niveles más altos de proteína c-reactiva a las 48 h pi y haptoglobina en plasma a partir de las 24 h pi. Tras realizar un test de supervivencia se asignó un mayor riesgo de sacrificio para hembras V que LP, durante las primeras horas después del desafío. Además, la ventaja en términos de tasa de supervivencia de la LP sobre la línea V procede exclusivamente de las hembras cubiertas después de la primera semana de destete (PW), que también era el grupo principal responsable del aumento de ácidos grasos no esterificados (NEFAs) en sangre después de 6 h pi. Estos resultados vuelven a confirmar que, respecto a la respuesta inmune frente a un desafío con LPS, la fundación por criterios de híper-longevidad productiva (hembras LP) conduce a obtener una población más robusta de conejas, que se caracteriza por la capacidad de una respuesta mejorada.

En conclusión, a partir de los resultados de estos tres estudios, se puede concluir que, parece haber una clara relación entre la condición corporal y el estado de salud de las conejas reproductoras, se han observado evidentes correlaciones positivas entre la condición corporal y los principales recuentos de poblaciones linfocitarias en los dos primeros estudios, y los niveles de NEFAs en plasma parecen ser el único parámetro en relación con la supervivencia de las conejas al desafío inmunológico desarrollado en el tercer estudio. Por lo tanto, estos resultados apoyan la hipótesis inicial de esta tesis, confirmando que la condición corporal desempeña un papel mediador fundamental. De tal forma que en animales bajo el mismo nivel productivo y el mismo nivel genético, a mejor condición corporal, mejor respuesta inmunitaria.

ABBREVIATIONS

AD Anno domini
Ag Antigen
AI Artificial insemination
AMP Antimicrobial peptide
AP-1 Activating protein 1
APC Antigen presenting cell
APP Acute phase protein
BALT Broncus-associated lymphoid tissue
BC Before Christ
Bcl-2 B cell lymphoma-2
BCR B-cell receptor
BLUP Best linear unbiased prediction
BM Bone marrow
BW Body weight
CC Climatic chamber
CCR CC-chemokine receptor
CD Cluster of differentiation
CH Conventional housing
CNS Central nervous system
CPK Creatine phosphokinase
CRP C-reactive protein
CSF Colony-stimulating factor
cTEC Cortical thymic epithelial cell
CTLA Antigen cytotoxic T lymphocyte-associated
CV Coefficient of variation
CXCL Chemokine (C-X-C motif) ligand
CXCR CXC-chemokine receptor
DC Dendritic cell
DE Digestible energy
DFI Daily feed intake
DM Dry matter
DN Doble negative
DP Doble positive
DPr Digestible protein
dpp Days post partum
EBE Estimated body energy
ECF Eosinophilchemotactic factor
EDTA Ethylenediaminetetraacetic acid
ERE Epizootic rabbit enteropathy
ETP Early thymic progenitor
FasL Fas ligand
Fc Fragment crystallizable
FcR Fc receptor
FoxP3 Forkhead box P3
GALT Gut-associated lymphoid tissue
GITR Glucocorticoid induced tumor necrosis factor receptor

GM-CSF Granulocyte-macrophage-colony stimulating factor
GOT Glutamic-oxaloacetic transaminase
GPT Glutamic-pyruvic transaminase
Hb Haemoglobin
H:L Heterophil/lymphocyte
Hp Haptoglobin
HPC Hematopoietic progenitor cell
HSC Hematopoietic stem cell
Hsp Heat-shock protein
HSPC Hematopoietic stem and progenitor cell
IEL Intraepithelial lymphocyte
IFN Interferon
Ig Immunoglobulin
IGF Insulin-like growth factor
IgH Immunoglobulin heavy chain
IgL Immunoglobulin light chain
IL Interleukin
iNOS Inducible nitric oxid synthase
IRF Interferon regulatory factor
iTreg Induced or adaptive T regulatory cell
KIR Killer cell immunoglobulin-like receptor
LA Leukocyte antigen
LCA Last control alive
LFA Lymphocyte function-associated antigen
LGL Large granular lymphocyte
LN Lymph node
LP Hyperlongevity productive rabbit line
LPS Lipopolysaccharide
LT Lymphotoxin
MAC Membrane attack complex
MALT Mucosa-associated lymphoid tissue
MASP Mannan-binding lectin-associated serine protease
MB Mannan-binding
MC Mast cell
MCH Mean corpuscular haemoglobin
MCHC Mean corpuscular haemoglobin concentration
MCP Monocyte chemotactic factor
MCV Mean corpuscular volume
MD2 Lymphocyte antigen 96
MHC Major histocompatibility complex
MIg Membrane immunoglobulin
MPS Mononuclear phagocytic system
mTEC Medullary thymic epithelial cell
MyD88 Myeloid differentiation primary response gene (88)
NADPH Nicotinamide adenine dinucleotide phosphate
NALT Nasopharynx-associated lymphoid tissue
NEFA Non esterified fatty acid
NET Neutrophil extracellular trap

NFAT Nuclear factor of activated T-cell
NF$_k$B Nuclear factor-kappa beta
NO Nitric oxide
No. Number of observations
NOD Nucleotide Oligomerization Domain
NK Natural killer
NKP Natural killer precursor
NKR Natural killer cell receptor
nTreg Natural T regulatory cell
PALS Periarteriolar lymphoid sheath
PAMP Pathogen-associated molecular pattern
PBS Phosphate-buffered saline
PCV Packed cell volume
PFT Perirenal fat thickness
pi Post-infusion
pMHC Peptide-major histocompatibility complex
PMN Polymorphonuclear neutrophil
PP Post-partum
PRR Pattern recognition receptor
pTα Pre-Tα chain
PW Post-weaning
RDW Red cell distribution width
RER Rough endoplasmic reticulum
RLA Rabbit leukocyte antigen
RNS Reactive nitrogen species
ROS Reactive oxygen species
SCA-1 Stem-cell antigen-1
SD Standard deviation
SE Standard error
SLO Secondary lymphoid organ
SP Single positive
SPF Specific pathogen free
TAP Transporter associated with antigen processing
Tc Cytotoxic T cell
TCR T-cell receptor
TD T-cell dependent
Tfh Follicular helper T cell
TGF Tumor growth factor
Th Helper T cell
TI T-cell independent
TIR Toll/interleukin-1 receptor
TIRAP Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR Toll-like receptor
TNF Tumor necrosis factor
Tr1 Regulatory T cell type 1
TRAF Tumor necrosis factor receptor associated factor
TRAM Toll-like receptor adaptor molecule
Treg $CD4^+CD25^+Foxp3^+$ regulatory T cell

TRG T-cell receptor gamma
TRIF TIR-domain-containing adapter-inducing interferon-β
UP Perirenal fat thickness at partum
V Reproductive rabbit line selected by litter size at weaning
WBC White blood cell

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GENERAL INTRODUCTION

A. GENERAL INTRODUCTION

1. RABBIT BREEDING

1.1. History of rabbit breeding

Considerable evidence suggests that Asia is the centre of origin of the mammalian superordinal clade Glires, which includes extant orders Rodentia and Lagomorpha (Rose *et al.,* 2008). The fossilized skeleton of a rabbit-like creature, which lived 55 million years ago, was found in Mongolia, *Gomphos elkema*, and as known, it is the oldest member of the rabbit family to have ever been found (Meng *et al.,* 2004). *Gomphos* shares a series of cranioskeletal characters with extant rabbits, hares and pikas, but retains primitive dentition and jaw as compared to its modern relatives. Phylogenetic analyses support the position of *Gomphos* as stem lagomorphs (Asher *et al.,* 2005). In Turkey, a sphinx has been found which rests on two rabbits dating back to the 14th century BC (**Figure 1**).

Figure 1. Sphinx gate of Alaca Hüyük in the 14th century BC. Ruler standing on a two-headed eagle catching two rabbits.

There are evidences to indicate that both wild and domestic European rabbits belong to the single species *Oryctolagus cuniculus*, which is the sole extant representative of the genus *Oryctolagus,* which appeared in the Iberian Peninsula, from where it went to West France and North Africa (Gibb, 1990; Mitchell-Jones *et al.,* 1999).

Paleontological data indicate a limited morphological diversification of the genus *Oryctolagus* since its appearance at the end of the Miocene (24 - 5 million years BC), where the oldest fossil was found near Granada (Rogel-Gaillard *et al.,* 2009). Fossil remains from the middle Pliocene helped recognize two species: *O. lacosti* in South France and North-West Italy, *O. laynensis* in the Iberian Peninsula. The latter is thought to be the origin of the extant *O. cuniculus* (Lopez-Martínez, 1989). Other *O. cuniculus* fossils dating back to the mid-Pleistocene (800,000-130,000 BC) come from Baza, Andalusia (South Spain) (Rogel-Gaillard *et al.,* 2009). Archaeozoological data indicate that the original range of rabbits was restricted to the Iberian Peninsula and to South France as far as the River Loire (Callou, 1995).

Nowadays, two subspecies (**Table 1**) have been recognized in the Iberian Peninsula (South Europe). *Oryctolagus cuniculus algirus* occupies the southwestern part of the peninsula

(roughly Portugal and South Spain). There is some overlap of ranges with *O. c. algirus* and *O. c. cuniculus*, which occupy all the northern and western points of *O. c. algirus* (Biju-Duval *et al.,* 1991). *O. c. cuniculus* is thought to be the descendant of the early domestic rabbits released into the wild (Gibb, 1990), and is now the subspecies that was introduced throughout Europe, and certainly worldwide (Angulo, 2004). *O. c. algirus* is also found in North Africa, in the Mediterranean region and on Atlantic islands (Branco *et al.,* 2000).

Table 1. Taxonomy of *Oryctolagus cuniculus* (Wilson and Reeder, 2005).

Domain	Eukaryota
Supergroup	Opisthokonta
Kingdom	Animalia
Clade	Eumetazoa
Subregnum	Bilateria
Group	Nephrozoa
Superphyla	Deuterostomia
Phylum	Chordata
Subphylum	Vertebrata
Infraphylum	Gnathostomata
Superclass	Tetrapoda
Class	Mammaliad
Subclass	Theria
Infraclass	Placentalia
Order	Lagomorpha
Family	Leporidae
Genus	Oryctolagus
Species	Oryctolagus cuniculus
Subspecies	***O. c. algirus*** - *O. c. brachyotus*
	O. c. cnossius - ***O. c. cuniculus***
	O. c. habetensis - *O. c. huxleyi*

The European rabbit (*Oryctolagus cuniculus*) has been introduced into many locations around the world, and all breeds of domestic rabbit originate from the European species. Nearly half the world's rabbit subspecies are in danger of extinction, and many are among the most vulnerable species of all mammals (Sandford, 1996). However, other subspecies have been widely spread, and are even considered a pest in some geographical areas. Currently, ranges include all Western European countries, Ireland and the UK (including all islands), Austria, parts of Sweden, Poland, the Czech Republic, Hungary, Romania, Ukraine, and the Mediterranean islands of Sicily, Corsica, Sardinia, Crete, the Balearics (Thompson and King, 1994), and also Croatia and Slovakia (Mitchell-Jones *et al.,* 1999). It was introduced into Australia in 1788, and again into South America in Argentina and Chile in 1859, and is found in many islands of the Pacific, off the African coast, New Zealand and the Caribbean (**Figure 2**) (Thompson and King, 1994).

The first news of the presence of rabbits in Spain is estimated to date back to 1,000 BC when the Phoenicians arrived to Iberia (Rogel-Gaillard *et al.,* 2009). Their attention was caught by a large amount of gray rabbits if compared to those that existed in their original place, and they called them "Damans", which were like guinea pigs, but bigger. In fact, during the last millennium BC, southern regions and areas of the Mediterranean coast of the Iberian Peninsula emphasized their relations with Eastern colonizers (Phoenicians and Greeks), while those inlands were influenced by Indo-Europeans (Celts), which entered via the Pyrenees.

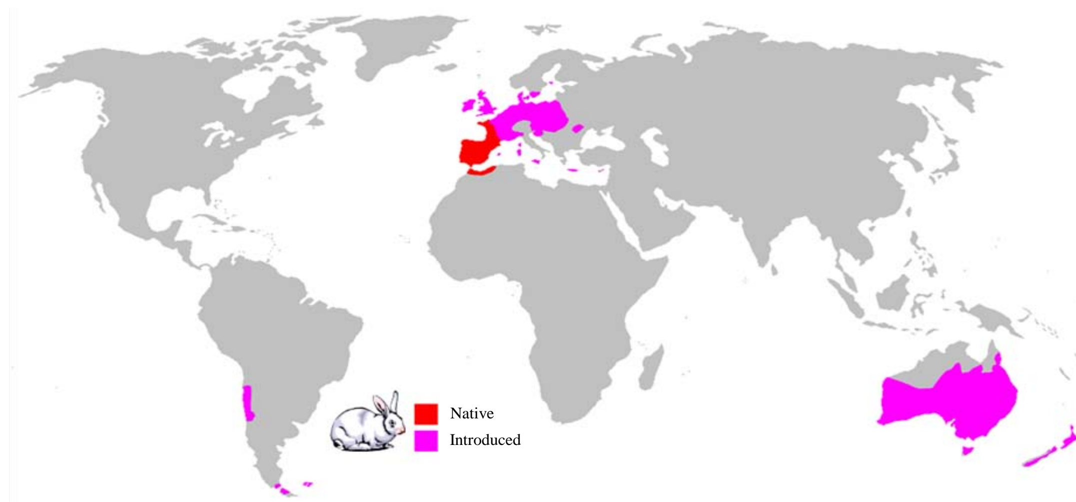


Figure 2. Distribution range map of *Oryctolagus cuniculus*, native or introduced (Linnaeus, 1758).

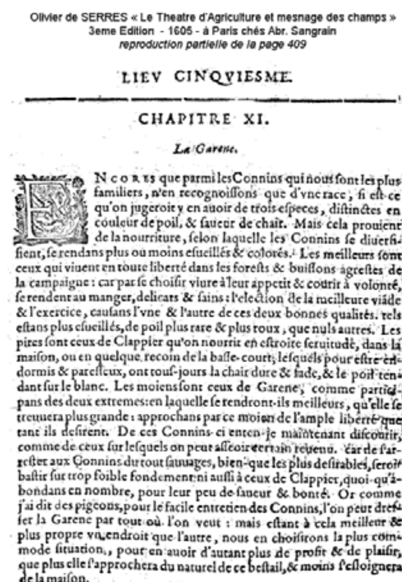
It is believed that the name of Spain derives from these animals. Damans in their language was *shapan*, for which the country was called *i-shaphan-im* or "the land of hyraxes", small rodents from their country, which were mistaken with rabbits (Slifkin, 2011).

Later, the Romans converted the phrase *i-shaphan-ím*, given an influence of Greek Spania, into its Latin form, *Hispania*, which evolved into the modern Spanish word "*España*" for Spain (Rogel-Gaillard *et al.,* 2009). In fact in the 2nd century BC, rabbits had been introduced from Spain into the Italian Peninsula (Hatcher and Battey, 2011). The Romans, therefore, gave Spain the meaning of "land rich in rabbits", as we can read in written records of Cicero, Caesar, Pliny, Cato, Livy, and, in particular, Catullus, referring to Hispania as "Cuniculi Peninsula" (on some coins minted at the time of Hadrian; 117-138 AD), including personifications of Spain as a lady with a rabbit sitting at her feet (**Figure 3**), referring to the time that he lived in Spain (Rogers *et al.,* 1994).

Figure 3. Second century Roman coins during the Hadrian Empire (117 to 138 BC). Dimensions: 19mm x 20mm, 7.12g. (Cuniculture: http://www.cuniculture.info/Docs/Elevage/Histori-02.htm).

During Roman times, the rabbit was still emblematic of Spain (Moreki, 2007), and it was present not only in Spain, but also in South France and North Africa (Gibb, 1990; Mitchell-Jones *et al.,* 1999). Rabbits had still not yet been domesticated, although Varro (116 to 27 BC) suggested that rabbits be kept in *leporaria,* stone-walled pens or parks, with hares and other wild species for hunting (Hatcher and Battey, 2011). These *leporaria* were the origin of the warrens or game parks that subsequently developed in the Middle Ages. In France, it became the sole right of the lord of the manor to keep warrens. Rabbits were scarcely hunted, and were captured with snares, nooses or nets (Lebas *et al.,* 1997). Pliny the Elder (23-79 BC), in his *Naturalis Historia*, wrote how this animal was used to prepare a delicacy based on fetal rabbits called *laurices*. Gregory of Tours (538-594 BC), in his *Historia Francorum* ("History of the Franks") Book V, 4, reported that Pope Gregory I authorized the use of *laurices* during Lent or other fasts, declared them to be a marine species, like fish or shellfish. This led to the development of cuniculture in monasteries during the early Middle Age, although breeding did not really take its roots in Europe until the 16th century (Carneiro *et al.,* 2011). Some authors suggest that rabbits were introduced later into Britain by the Romans, who kept them in fenced-off warrens and harvested their meat and fur. The earliest known written records on rabbits in Britain date back to the 12th century. They were first described as "conies" after the second part of their scientific name *Oryctolagus cuniculus* (Bixby and Whitman, 2005).

During the 16th century, breeding seemed to have spread across to France, Italy, Flanders and England. In 1595, Agricola mentioned the existence of gray-brown (wild), white, black, piebald (black and white) and ash-gray rabbits. In 1606, Olivier de Serres classified three types of rabbit: the wild rabbit, the semi-wild or "warren" rabbit raised inside walls or ditches, and the domesticated or hutch-bred rabbit (**Figure 4**). The meat of the last type was described as insipid and that of the wild- or semi-wild type as delicate. Domestication began in France since 1700, where a wild rabbit, called Agouti, was known to be brown, white, yellow, black and spotted (Lebas *et al.,* 1997).



Olivier de SERRES « Le Theatre d'Agriculture et mesnage des champs »
3eme Edition - 1605 - à Paris chés Abr. Sangrain
reproduction partielle de la page 409

LIEV CINQVIESME

CHAPITRE XI.

La Garene.

ENCORES que parmi les Connins qui nous sont les plus familiers, n'en recognoissons que d'vne race, si est-ce qu'on jugeroit y en auoir de trois especes, distinctes en couleur de poil, & saueur de chair. Mais cela prouient de la nourriture, selon laquelle les Connins se diuersifient, se rendans plus ou moins esueillés & colorés. Les meilleurs sont ceux qui viuent en toute liberté dans les forests & buissons agrestes de la campaigne : car par se choisir viure à leur appetit & courir à volonté, se rendent au manger, delicats & sains : l'election de la meilleure viáde & l'exercice, causans l'vne & l'autre de ces deux bonnes qualités. tels estans plus esueillés, de poil plus rare & plus roux, que nuls autres. Les pires sont ceux de Clappier qu'on nourrit en estroite seruitude, dans la maison, ou en quelque recoin de la basse-court, lesquels pour estre endormis & paresseux, ont tous-jours la chair dure & fade, & le poil tendant sur le blanc. Les moiens sont ceux de Garene, comme participans des deux extremes: en laquelle se rendront-ils meilleurs, qu'elle se treuuera plus grande : approchans par ce moien de l'ample liberté que tant ils desirent. De ces Connins-ci enten-je maintenant discourir, comme de ceux sur lesquels on peut asseoir certain reuenu. car de s'arrester aux Connins du tout sauuages, bien-que les plus desirables, seroit bastir sur trop foible fondement: ni aussi à ceux de Clappier, quoi-qu'abondans en nombre, pour leur peu de saueur & bonté. Or comme j'ai dit des pigeons, pour le facile entretien des Connins, l'on peut dresser la Garene par tout où l'on veut : mais estant à cela meilleur & plus propre vn endroit que l'autre, nous en choisirons la plus commode situation, pour en auoir d'autant plus de profit & de plaisir, que plus elle s'approchera du naturel de ce bestail, & moins s'esloignera de la maison.

Figure 4. The starting chapter devoted to Garenne in the work of Olivier de Serres (1605) entitled "The Theatre of Agriculture and Husbandry of Fields".
(Cuniculture: http://www.cuniculture.info/Docs/Elevage/Histori-02.htm).

From Spain, the rabbit moved to America after being introduced by conquerors. Introduced into South America unsuccessfully several times since the mid-nineteenth century, but successfully at around 1936 where it maintains limited ranges in Argentina and Chile. Found on many islands in the Pacific, off the African coast, New Zealand, and the Caribbean (Thompson and King, 1994).

During the 19th century, society profoundly changed, especially in Europe. After the abolition of aristocratic privileges and hunting practices, rabbit breeding in farms developed in Western Europe in both rural and urban areas. This period saw the beginning of what was to become practiced selective breeding. Numerous rural people started to migrate to work in new urban industries. In the small gardens often attached to their homes, the new workers started to breed poultry and rabbits. Indeed these animals allowed the development of kitchen by-products and became part of crop production in small gardens (**Figures 5**). Rabbits were then reared in small cages in a room annexed to the house, and even in the dwelling itself, as evidenced by several paintings from that time (Lebas *et al.,* 1997).

Figures 5. Children feeding rabbits in front of their cages. **a**: Table J. F. Herring, mid-19th century; **b**: Table Felix Schlesinger, late 19th century; **c**: Hutchin rural areas. Girl holding a rabbit-Detail of a Table J. L. Krimmel painted in 1812; **d**: Merchant of rabbits (a mother and her cubs) – Engraving by H. Wolf, late 19th century, U.S. (Cuniculture: http://www.cuniculture.info/Docs/Elevage/Histori-02.htm).

In the 20th century, with the development of more intensive rabbit production, the first feed concentrates appeared. During World War II, rabbit production developed extensively throughout Europe and Japan to cope with meat shortages. Under these demanding conditions, rabbits demonstrated their highly efficient feed-conversion capacity. In the 1950s, production slumped in Japan and North European countries as other meats with more flavour became readily available. At the same time, a boom in the New Zealand White rabbit and its offshoot, the Californian rabbit, was seen. Traditional European breeds (Fauve de Bourgogne, Argenté de Champagne and French Belier) underwent regression. French and

Italian breeders worked to substantially improve the first New Zealand White and Californian rabbits imported from the United States.

In Spain, different genetic lines have been set up, which have been exported to several countries. Their attractive appearance and quiet manner have made domestic rabbits good, relatively undemanding pets. As they are easily raised in captivity, rabbits are also important as laboratory animals for medical and scientific purposes (Sandford, 1996). Other quantitatively minor activities include rabbit breeding for experimentation purposes, for example, in the pharmaceutical industry, rabbit hair production (Angora rabbits), furs (Orylag Rex rabbits), as well as domestic rabbits (Rosell *et al.,* 2000b).

1.2. Rabbits in the world and European context

Global production rose roughly from 1975 to 1990 (Lebas *et al.,* 1997). An estimate of rabbit meat production (1994) by Colin and Lebas, including almost all the countries in the world, suggests a possible production of 1.5 million tons. This means a per capita input of annual consumption of roughly 280 g of rabbit meat, if compared against the 10 kg/year consumed in France and the 15 kg/year in Naples, Italy (Lebas *et al.,* 1997). Besides, FAOSTAT (2012) reports other, revealing that world rabbit meat production in the same year amounted to 883,796 tons, nearly half that declared by previous authors.

Industrial rabbit production started to develop in Europe in the late 1970's; in the 1980s, rabbitries incorporated a series of techniques that allowed production to improve. In the 1990s, specialized industrial rabbit meat production based on "new" genetic rabbit lines developed, and farmers were assisted by specialists in business management, nutrition, pathology and genetics, only to become highly competitive farms compared to other farmers exploiting different species of meat-producing animals.

According to the data provided by the FAOSTAT (2012), world rabbit meat production has steadily grown since the late 1990s, reaching 1,136,305 tons in 2004, representing a 13% increase as of 1998. China remained the largest producer, accounting for 41% of total production in 2004. It should be noted that the entrance of rabbit meat from this source into the European Union was banned in 2002. This measure emerged after the detection of serious deficiencies in the control of waste and use of veterinary products from China, which is not allowed by the Standing Veterinary Committee of the EU. Since then, the measure has not been relented. In 2004, other major producing countries to consider are Italy with 18%, the Democratic People's Republic of Korea with an average of 8%, and Spain with 6%. World rabbit meat production since 2005 has ranged from 1,191,255 to 1,401,081 tons in 2009 (FAOSTAT, 2012).

The latest FAOSTAT data of 2010 indicate that rabbit meat production rose to 1,421,695 tons worldwide, which is a similar trend to 2009 in each country. According to this source, the evolution of global rabbit meat production in the last ten years has grown until 2007 to then lower and stabilize in recent years, presenting a similar trend of world meat production (**Figure 6**). Asia with 809,071 tons remains the largest producer, where China generates 669,000 tons, accounting for 47% of global rabbit meat production, followed by Europe as Italy generates 255,400 tons (**Figure 7**).

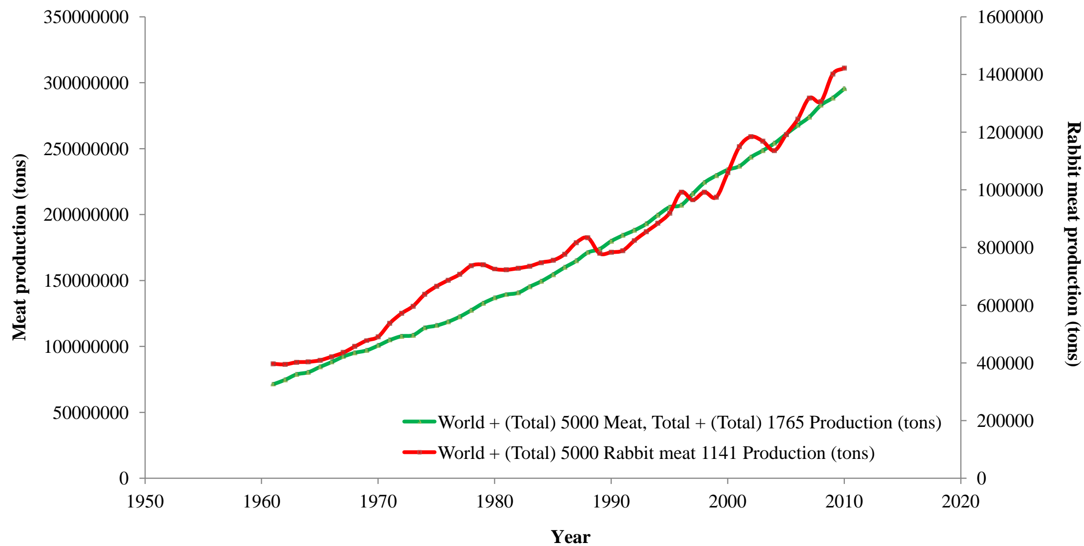


Figure 6. Evolution of world rabbit meat production (tons) *vs*. evolution of world meat production (tons) (FAOSTAT, 2012).

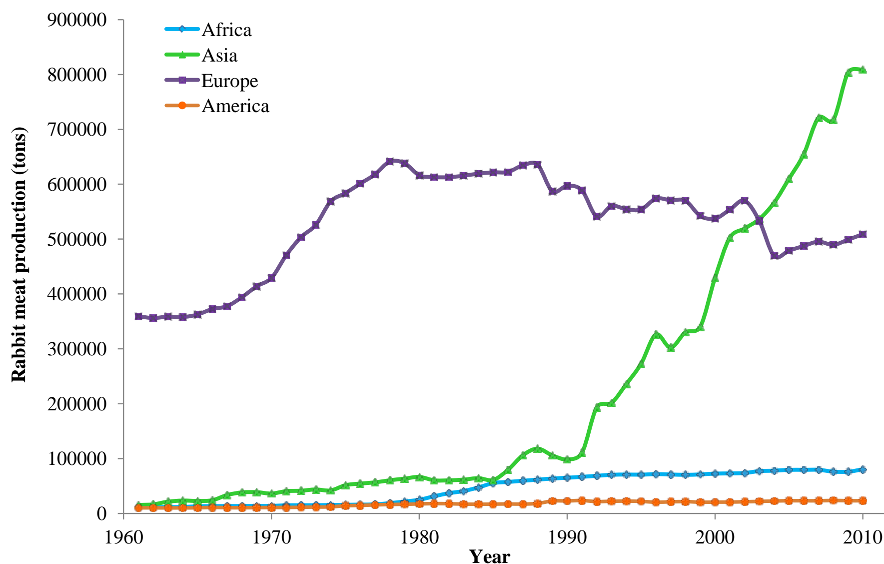


Figure 7. Evolution of world rabbit meat production by continents (tons) (FAOSTAT, 2012).

Overall, worldwide rabbit meat production has remained constant in recent years, but mainly thanks to increased production in China. Of the one million, four hundred thousand tons, 509,107 tons correspond to the EU. Of this figure, Italy is the largest producer, accounting for 50% of European production in 2010.

The Italian rabbit meat production increased incessantly at the expense of Hungary. The second country that stands out for its rabbit meat production is Spain with 66,200 tons, whose production remains constant over the years, be it with oscillations. France with 51,665 tons ranks third place, although its production tends to decrease (**Figure 8**). Beyond the EU, Egypt is highlighted (69,840 tons) as maintaining high production, which remains similar to that of 2003 (FAOSTAT, 2012).

In its directory, FAO data (2005) indicate that Italy presents the highest rabbit meat consumption per capita per year in Europe at 3.84 kg. It is followed by Czech republic (3.82 kg), Republic of Malta (3.69 kg), Spain (2.5 kg), France (1.37 kg), Cyprus (1.03 kg), Greece (0.55 kg), Germany (0.45 kg) and, finally, Hungary (0.23 kg).

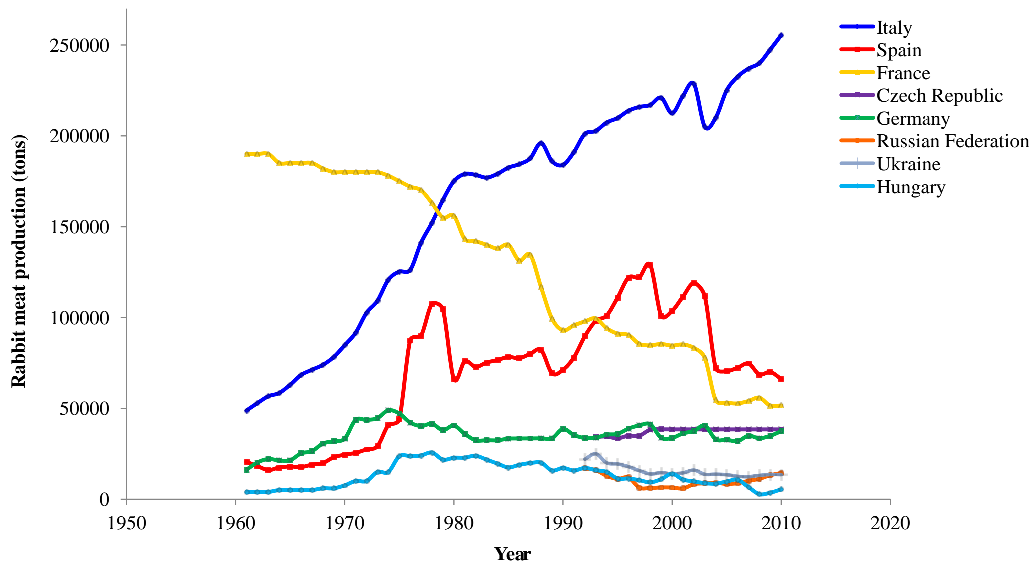


Figure 8. Evolution of European countries rabbit meat production (tons) (FAOSTAT, 2012).

According to world rabbit meat production figures (FAOSTAT, 2012), the evolution of the number of slaughtered producing animals recorded in recent years was similar. In 2004, the number of producing animals slaughtered around the world was of 817,779,000 animals. In 2005, 865,457,000 were recorded, while in 2007 this number was 951,935,000 animals. A slight decrease was recorded in 2008, although it increased again in 2009, with the same number of animals in 2010 (1,050,641,000 animals), and the country with the largest number of animals recorded was China with 514,600,000, representing 50% of the global amount. In Europe, 325,106,000 animals were recorded in 2010, representing 31% of the global number of producing animals slaughtered, for which Italy leads with 169,700,000 animals, followed by Spain with 58,800,000 and France with 36,777,000.

1.3. Rabbits in the national context

In Spain, rabbit breeding started to be modernized in the 1950s, and continued to evolve into the 1960s and 1970s. Industrial rabbit production began in Spain in the late 1960s and the early 1970s. It progressed from raising rabbits on the ground in niches or wooden cages placed in pens, townhouses in exterior walls or stalls, to exploiting them in metal cages located outdoors, either under covers or in rudimentary or empty rooms.

Rabbit breeding was a largely rural, family activity. Fed mainly on green fodder and domestic food scraps, rabbits underwent enormous productive changes throughout the year, with slow, irregular growth, and could cause major health problems (Roca, 2009). Today, rabbits are exploited economically for meat production, and also for skin and hair production, although this latter exploitation is often a byproduct in most cases. Rabbits are

also kept as pets (dwarf breeds), and used for experimentation purposes and for carrying out reforestation hunting (wild rabbits).

During the early years of industrial rabbit production in Spain, rabbit meat production represented a business opportunity. Rabbit breeding started to move toward an industrialization and modernization process based on animal breeding, feed formulation and health plans. Rabbit meat production increased from 24,500 tons in the year 1970 to 69,279 tons in the late 80's to reach the full production in 1997, being 122,181 tons. A number of advances were also recorded at the time: medium farms became large-sized ones and small ones disappeared, and animals were housed in metal cages with equipping management, plus holding cages with nesting, females productive labor and the artificial insemination technique.

In 1997, with the emergence of Epizootic Rabbit Enteropathy, domestic production was severely affected by the presence of this new disease, which destabilized almost all the farms in Spain (Licois *et al.,* 2000). In fact, FAO statistics estimate a decline in production in 2000 of 15% if compared to 1997, which led farmers to alarming situations as a result of this disease and to modifying traditional management protocols in an attempt to overcome this serious problem.

Since late 2004, rabbit breeding in Spain has remained in a situation of crisis, which became particularly acute in 2007 when, according to data compiled by the Spanish Ministry of Environment and Rural and Marine Affairs (2010a), a crisis broke out in which prices and the increased the price of grain for feed were influencing factors. It was then when the poor market situation of rabbit skin was exacerbated following the closure of the Chinese market, the traditional destination for tanning. Production as of this year ranged from 70,000 tons of meat per year (**Figure 8**).

According to the census data published in the latest National Survey of Rabbit (2008), the national census amounted to 1,221,495 breeding females, with a total of 6,903,718 animals for both genders and age in 2007. In terms of Spanish regions, the Valencian Community ranked third, after Catalonia and Aragon with 158,459 females. This drop in the number of animals was between 16-20% as compared to the results of the 2004 survey, where the national census stated 1,458,387 breeding females and a total of 8,563,791 animals.

The Spanish Ministry of Environment and Rural and Marine Affairs (MARM) data confirm that the rabbit meat-producing sector in Spain has remained in a crisis situation for years. The number of farms has decreased dramatically (54%) between 1993 and 2007. This crisis has been coupled with a significant decrease of 33.87% in the rate of average rabbit meat consumption per capita/year from 1999 to 2009 for Spain (Kallas and Gil, 2012). In 2009, the number of farms dropped by 11%, and by the end of the year, there were 4,090 farms. Latest data provided by Spanish Ministry of Agriculture, Food and Environment (MAGRAMA, 2011) reported numbers also dropped drastically in 2011, 3,806 farms were recorded on April 2011.

Traditionally, the balance of foreign trade in the Spanish rabbit sector has been generally positive. However in recent years, the absolute stock value has gradually lowered. In 2009, 610 tons were imported (491 EU and other third world countries) and 2220 tons were exported (1883 to EU countries). Both exports and imports to/from the EU dropped. Rabbits

are annually imported, especially from France, and are also exported to neighboring countries where rabbit meat production sometimes proves insufficient to cover its own consumption. The evolution of both Spanish rabbit meat production and the number of Spanish producing animals slaughtered in the last ten years increased until 2002, after which it lowered until 2004 and has remained constant to the present-day (**Figure 9**).

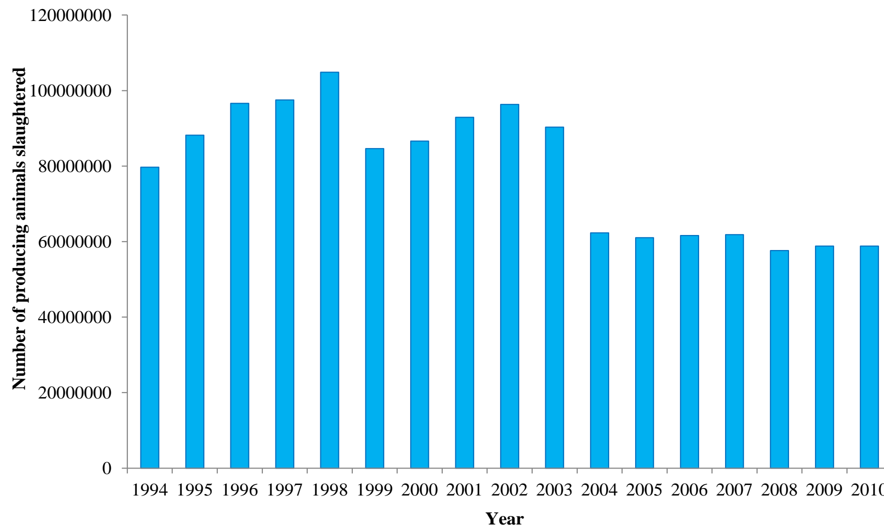


Figure 9. Evolution of the number of Spanish producing animals slaughtered (FAOSTAT, 2012).

As this section describes, the main weakness in modern rabbit production systems are problems related to rabbit health, which is probably also the main reason of farms closing through loss of competitiveness that this entails. Digestive troubles are the most important causes of losses (about 25% around weaning; i.e., 18-50 days of age) and the low welfare-status in rabbit breeding throughout Europe. Consequently, antibiotherapy is extensively used to improve the digestive security of rabbits. However, the second main pathology problem in rabbit breeding relates to does' health status, mainly linked with deficient corporal status and to intense reproduction rhythm, which could lead to large replacement rates (Rosell and de la Fuente, 2009) and worse general health on rabbit farms (Pascual *et al.,* 2012). More effort made to improve knowledge on the female rabbit immune system and how management, genetic and feeding practices can affect the immune response of the females could help reduce the weaknesses of rabbit production linked to health.

2. IMMUNE SYSTEM

2.1. Introduction

All organisms have mechanisms to defend against possible attacks by pathogens and foreign invasions. On the phylogenetic scale, these defence mechanisms significantly differ on the phylogenetic scale, with a tendency towards greater complexity and power of defense responses, and also towards the specific recognition of the pathogenesis of the most evolved species.

The immune system has evolved to protect the host against the attack of foreign, potentially pathogenic, microorganisms. It does so by recognizing the antigens expressed by those microorganisms and by developing an immune response against all cells expressing them, with the ultimate aim of their elimination (O`Garra and Vieira, 2004).

The first defense mechanism, and the most effective, is to prevent entrance of pathogens through the development of physical (such as skin and the mucosal surfaces) and/or chemical barriers (pH and various soluble factors, such as lysozyme interferon and complement). Such barriers are found in all living species and have unique characteristics depending on which organism is concerned.

Along with these barriers, animals, invertebrates and vertebrates have a second level of complexity, which presents an immune system responsible for defense. In the case of invertebrates, they have only an innate immune system, which involves cellular components, (phagocytic cells) and soluble or humoral components.

Only vertebrate animals have a third level of complexity, called an adaptive or acquired immune system, possessing T and B lymphocytes, and antibodies. Approximately 500 million years ago, two types of recombinatorial adaptive immune systems appeared in vertebrates (Panzer and Cooper, 2006). Among the key features setting them apart from invertebrate animals, vertebrates have the ability to not only specifically recognize an antigen (due to the wide variety of different receptors on the surface of T and B lymphocytes), but to recall previous exposures to antigen (memory) and to respond more effectively after getting back in touch with the same antigen (maturation of the immune response). In short, the infection site and type of pathogen largely determine which immune responses will be effective (Roitt *et al.*, 1996).

2.2. Organization of the rabbit immune system

Immune system development differs in various animal species (Jeklova *et al.,* 2007b). At birth, rabbits are physiologically immature, hairless, and open their eyes on day 10 of life (Sterzl and Silverstein, 1967). Their lymphoid organs are not fully developed. First, lymphatic follicles start to form at the age of 2 weeks. During maturation, the numbers of follicles increase (Thorbecke, 1960; Jeklova *et al.,* 2007a) and, moreover, lymphocyte subset distribution in spleen, lymph nodes and the peripheral blood of rabbits undergoes noticeable postnatal development. In any case, the rabbit's lymphoid system is generally organized in the same way as that of other mammals (Drouet-Viard and Fortun-Lamothe, 2002).

Lymphoid organs are classified as either primary or secondary organs (Roitt *et al.,* 1996). Primary organs are the bone marrow and thymus, and are responsible for the production and

maturation of lymphocytes. Secondary organs include the spleen, Peyer's patches, the appendix, tonsils and lymph nodes. They are responsible for further maturation of lymphocytes and initiation of an immune response (Weih and Caamaño, 2003). The bone marrow, thymus gland, fetal liver and the vermiform appendix are primary organs in rabbits. Thus, they acquire their repertoire of specific antigen receptors to cope with the antigenic challenges received during their lifespan (Roitt *et al.,* 1996).

Bone marrow (BM) resides within the medullary cavity of bones (Del Fattore *et al.,* 2010). It is a network of connective tissue fibers, fat cells, blood vessels and blood-producing cells. BM produces both red and white blood cells, including lymphocytes. Both T and B lymphocytes are produced in BM. Young T-cells move to the thymus for final development, but B-cells remain in BM during maturation. At this time, IL-7 is secreted by stromal cells, which stimulate the growth and survival of B-cells and T-cells. Chemokine CXCL12 is responsible for early B-cell development stages (Janeway *et al.,* 2001). This process is named lymphopoiesis and refers to the production of new lymphocytes (Snyder, 2012). Once B-cells have fully developed in BM, they are also released into circulation and the majority are located in secondary lymphoid organs. There are interspecies variations in the B-cell development and repertoire generation processes, which contrasts with the greater consistency of T-cell development. B-cell development in mice and humans, with postnatal B-cell generation of a new repertoire in BM throughout life, is regarded a 'standard' pattern; however in other species, the gut-associated lymphoid tissue (GALT) works as the primary lymphoid organ (Alitheen *et al.,* 2010).

The mammalian **thymus** arises as bilateral structures from the third pharyngeal pouch in the embryonic foregut (Manley, 2000; Boehm *et al.,* 2003). On the basis of functional and epithelial components, three zones are recognized: a subcapsular zone, cortex and medulla (Fry and Donald McGavin, 2012) (**Figure 10**).

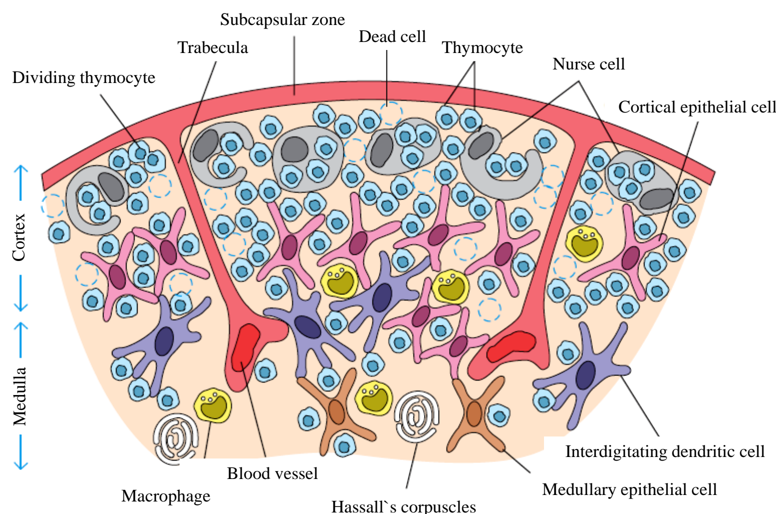


Figure 10. Diagrammatic cross section of a portion of the thymus, showing several lobules separated by connective tissue strands (trabeculae). The densely populated outer cortex is thought to contain many immature thymocytes (blue), which undergo rapid proliferation coupled with an enormous rate of cell death. Also present in the outer cortex are thymic nurse cells (gray). The medulla is sparsely populated and is thought to contain thymocytes that are more mature. During their stay within the thymus, thymocytes interact with various stromal cells, including cortical epithelial cells (light red),

medullary epithelial cells (tan), interdigitating dendritic cells (purple), and macrophages (yellow). These cells produce thymic hormones and express high levels of class I and class II MHC molecules. Hassalls corpuscles, found in the medulla, contain concentric layers of degenerating epithelial cells (Goldsby *et al.,* 2003).

Thymus size is variable and depends on age. The thymus undergoes its maximum development at birth (Varga *et al.,* 2011), but begins to diminish after sexual maturity. However, it is conceivable that T-cell generation within the thymus continues into adult life, albeit at a low rate (Roitt *et al.,* 1996).

The thymus is a primary or central lymphoid organ, which educates T-cells to identify foreign cells in the body, such as invading bacteria, and to mark them for destruction by other immune cells. T-cells precursors proliferate and mature in the thymus gland and learn to distinguish between self- and non self cells. Therefore, it plays an important key role in cell-mediated immunity (Dorko *et al.,* 2011). Moreover it performs an important endocrine function by secreting thymic hormones involved in T lymphocytes development and antibody production (Roitt *et al.,* 1996).

At an early stage, T-cells precursors have both lymphoid and myeloid potential (Bleul and Boehm, 2000; Liu *et al.,* 2005), and are characterized by the expression of CXC-chemokine receptor 4 (CXCR4) and CC-chemokine receptor 9 (CCR9) which, along with CCR7, play a central role in this precocious thymus colonization stage. In this differentiation stage, lymphoid cells also express stem- and progenitor-cell markers KIT (also known as CD117) and stem-cell antigen-1 (SCA-1) (Love and Bhandoola, 2011) (**Figure 11**).

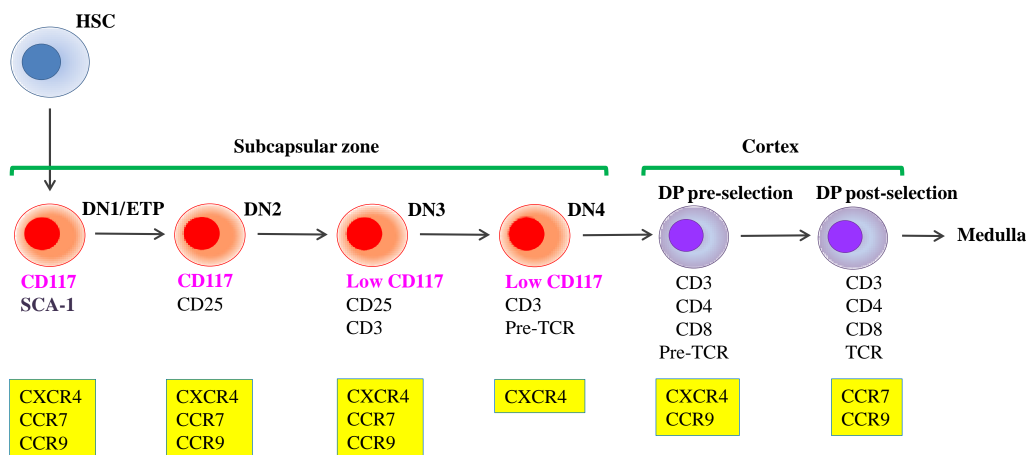


Figure 11. Chemotactic receptors expressed by progenitors of thymocytes. *CCR*: CC-chemokine receptor; *CXCR*: CXC-chemokine receptor; *DN*: Doble negative; *DP*: Doble positive; *ETP:* Early thymic progenitor; *HSC*: Hematopoietic stem cells; *TCR:* T-cell receptor.

Bone marrow-derived T lymphocytes enter circulation, travel to the thymus and enter the subcapsular zone through the corticomedullary junction (Fry and Donald McGavin, 2012). The intrathymic development of T-cells consists of several phases which require a dynamic relocation of developing lymphocytes within the multiple architectural structures of this organ (Romano *et al.,* 2012). In the subcapsular zone, lymphoid progenitor cells begin differentiation and selection processes, and develop into more mature naive T-lymphocytes as they traverse the thymic cortex to the medulla. In the cortex, the T lymphocytes that

recognize self-molecules (i.e., major histocompatibility complex molecules), but not self-antigens, are permitted to mature by a process called *positive selection*. When both have been recognized, they are removed by macrophages at the corticomedullary junction during a process called *negative selection*. The cells that do not recognize major histocompatibility complex molecules (MHC) are removed by apoptosis (Fry and Donald McGavin, 2012), so they cannot harm the body.

Traditionally, the developmental pathway is divided into three subsequent steps, as defined by peculiar immunophenotypic patterns: the $CD4^{-}CD8^{-}$ double negative (DN) stage; the $CD4^{+}CD8^{+}$ double positive (DP) stage; the $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{-}$ single positive (SP) stage (Romano *et al.,* 2012).

As seen in **Figure 12**, these steps are: (1) entry of lymphoid progenitor cells into the thymus; (2) generation of $CD4^{+}CD8^{+}$ double positive (DP) thymocytes in the cortex; (3) positive selection of DP thymocytes in the cortex; and (4) interaction of positively selected thymocytes with medullary thymic epithelial cells to complete thymocyte maturation and to, eventually, export mature T-cells from the thymus (5) (Romano *et al.*, 2012). After maturing in the thymus, mature naive T lymphocytes leave the thymus through postcapillary venules in the corticomedullary region, enter the circulation and recirculate through secondary lymphoid tissues in a highly regulated manner (Fry and Donald McGavin, 2012), where most remain to become mature immunocompetent T lymphocytes.

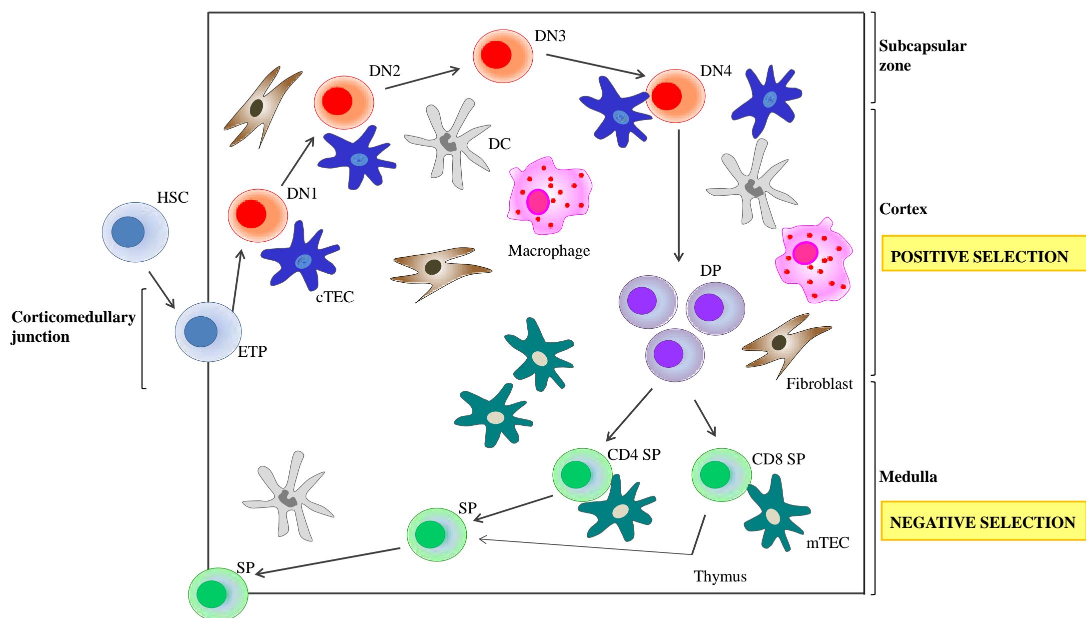


Figure 12. Steps of T-cell development in humans. The lymphoid progenitor cell enters the thymus through the corticomedullary junction. DN thymocytes ($CD4^{-}CD8^{-}$) migrate across the subcapsular region and then the outer cortex. Interaction between DN cells and cTECs generates DP thymocytes ($CD3^{+}CD4^{+}CD8^{+}$). Positively selected thymocytes interact with mTECs to complete the maturation process. In the medulla, self-reactive thymocytes are deleted, SP ($CD3^{+}CD4^{+}$or $CD3^{+}CD8^{+}$) thymocytes are generated and, eventually, the mature T-cells from the thymus are exported. *cTECs*: Cortical thymic epithelial cells; *DC*: Dendritic cell; *DN*: Doble negative; *DP*: Doble positive; *ETP*: Early thymic progenitor; *HSC*: Hematopoietic stem cells; *mTECs*: Medullary thymic epithelial cells; *SP*: Single positive (Adapted from Romano *et al.,* 2012).

In fetuses, most lymphocyte development occurs in the fetal liver for B-cells and in the thymus for T-cells. Many studies have hypothesized that adult and fetal human T-cells differ because they derive from distinct populations of multilineage hematopoietic stem cells (Zanjani *et al.,* 1993; Morrison *et al.,* 1995; Harrison *et al.,* 1997; Montecino-Rodriguez *et al.,* 2006). Mold *et al.* (2010) support the hypothesis that hematopoiesis occurs in distinct waves, including an early one that is fetal and a later one that is adult, with each generating a distinct populations of cells that may co-exist for a period of time (**Figure 13**).

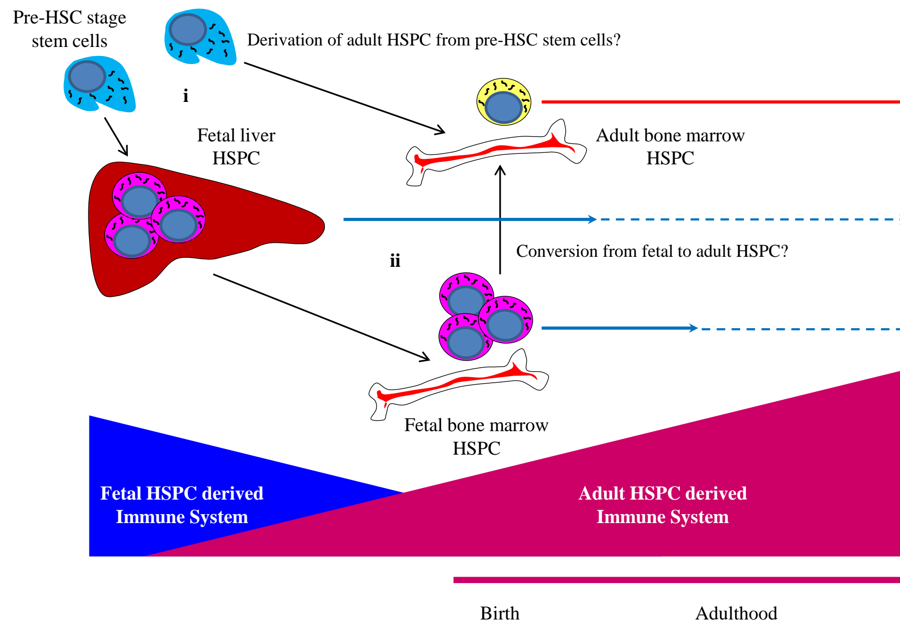


Figure 13. Human model for the transitions between fetal and adult hematopoiesis. There are two predominant models that could account for the switch from fetal to adult hematopoiesis (i) the generation of separate hematopoietic stem cells (HSC) or progenitor cells from a single precursor population that exists upstream of the identified HSC populations; (ii) a transition directly from existing fetal HSC to adult HSC triggered by changes in the environment in which they reside. In each case, it is possible that small populations of fetal HSC persist throughout life (dashed lines) which may contribute to low levels of "fetal" hematopoiesis. *HSPC*: Hematopoietic stem and progenitor cells (Mold *et al.,* 2010).

In rabbits, together with the fetal liver, there is another primary lymphoid organ belonging to the intestine located at the end of the caecum, a kind of **vermiform appendix** responsible for the production and maturation of B-cells during the first weeks of life (Reynaud and Weill, 1996; Pospisil and Mage, 1998).

In young rabbits, the vermiform appendix develops a primary lymphoid organ role, while in growing animals, it intervenes in the induction of specific immune responses as a secondary lymphoid organ (Drouet-Viard and Fortun-Lamothe, 2002). B-cells, which differentiate in the appendix in contact with intestinal flora (Fuschiotti *et al.,* 1997; Lanning *et al.,* 2000a), migrate to other parts of the intestine, particularly to Peyer's patches. The appendix is essential for the diversification of the antibody repertoire and for the development of the mucosal immune system (Dasso and Howell, 1997; Vajdy *et al.,* 1998). In their book, Rolleston and Hatchett Jackson (1888) provide a simple illustration of the caecum with a vermiform appendix and parts of the large and small intestines of rabbit (**Figure 14**).

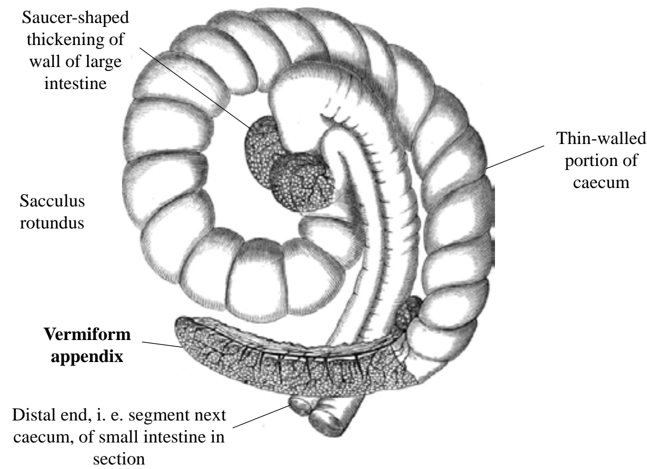


Figure 14. The caecum with vermiform appendix and parts of large and small intestine of rabbit (Rolleston and Hatchett Jackson, 1888).

After maturation and differentiation, lymphocytes migrate from primary lymphoid organs to **secondary lymphoid organs (SLOs),** which include spleen, lymph nodes and aggregated lymphoid tissue (**Figure 15**) (Drouet-Viard and Fortun-Lamothe, 2002).

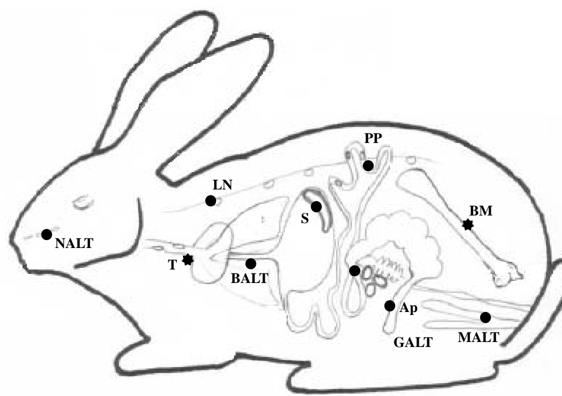


Figure 15. Distribution of primary and secondary lymphoid organs in the rabbit. ✸ Primary lymphoid organs: Bone marrow (BM), Thymus (T), Fetal liver, Vermiformis appendix (Ap); ● Secondary lymphoid organs: Spleen (S), Lymph nodes (LN), Mucosa associated lymphoid tissues [intestinal and urogenital] (MALT), Gut associated lymphoid tissues [Vermiformis appendix, Peyer´s patches (PP)] (GALT), Nasal associated lymphoid tissues (NALT), bronco-alveolar lymphoid tissues (BALT) (Drouet-Viard and Fortun-Lamothe, 2002).

SLOs develop during embryogenesis or during the first weeks after birth according to a highly coordinated series of interactions between newly emerging hematopoietic cells and immature mesenchymal or stromal cells. These interactions are orchestrated by homeostatic chemokines, cytokines and growth factors that attract hematopoietic cells to future lymphoid organ development sites, and they promote their survival and differentiation (Randall *et al.,* 2008). SLOs are strongly connected to blood, thus facilitating cell-cell interactions across the entire body. Interactions between naive T-cells and antigen-presenting cells (APCs), such as dendritic cells (DC), are particularly important for immune responses (Graw and Regoes, 2012). Therefore, these highly organized SLOs provide the structures where an antigen is

efficiently retained and presented, and where the ordered cellular interactions among APCs, T-cells and B-cells take place to initiate and promote efficient immune responses (Paul, 1993).

The **spleen** is a lymphatic organ that plays a fundamental role in protecting the body from invading pathogens (Tiron and Vasilescu, 2008). It is composed of two types of tissue: red pulp and white pulp (**Figure 16**). Red pulp is a blood filter that removes foreign material and damaged erythrocytes (Cesta, 2006). It is mostly used to also store blood and to destroy old red blood cells. The spleen is also the largest SLO and has evolved a unique structure to sample blood-borne antigens (Randall *et al.,* 2008). This function is charged to white pulp which surrounds central arterioles (Cesta, 2006). In fact, it combines the innate and adaptive immune system in a unique way by releasing an immediate innate reaction to microbial penetration, but also an adaptive immune response that involves the interaction of those cells which recognize a particular antigen, implicating the MHC molecules presented by antigen-presenting cells (Tiron and Vasilescu, 2008).

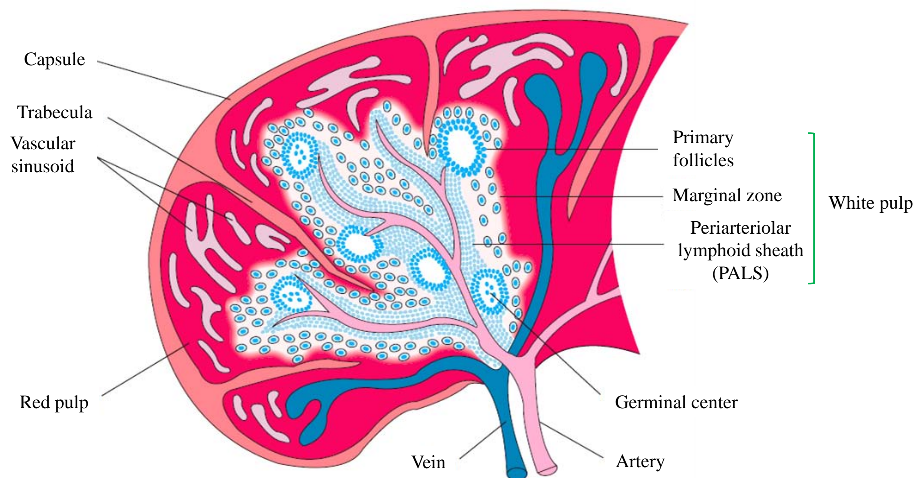


Figure 16. Diagrammatic cross section of the spleen. The splenic artery pierces the capsule and divides into progressively smaller arterioles, ending in vascular sinusoids that drain back into the splenic vein. The erythrocyte-filled red pulp surrounds the sinusoids. The white pulp forms a sleeve, the periarteriolar lymphoid sheath (PALS), around the arterioles; this sheath contains numerous T-cells. Closely associated with the PALS is the marginal zone, an area rich in B-cells that contains lymphoid follicles that can develop into secondary follicles containing germinal centers (Goldsby *et al.,* 2003).

Lymph nodes (LNs), like the spleen, have a highly organized architecture with different anatomical compartments for specific subsets of lymphocytes (Graw and Regoes, 2012). LNs consist of multiple lymphoid lobules surrounded by lymph-filled sinuses enclosed by a capsule (Willard-Mack, 2006). LNs consist in anatomic components such as stroma (fibrous capsule and reticulum), the cortex (the outer cortex containing B lymphocytes and the deep cortex containing T lymphocytes), medulla (medullary cords containing macrophages, lymphocytes and plasma cells surrounding medullary sinuses), vasculature and the cells of the monocyte-macrophage system (Fry and Donald McGavin, 2012). Both B- and T-cells are located in separate areas within these compartments, and interact with antigen-presenting cells to undergo clonal expansion (Willard-Mack, 2006) (**Figure 17**).

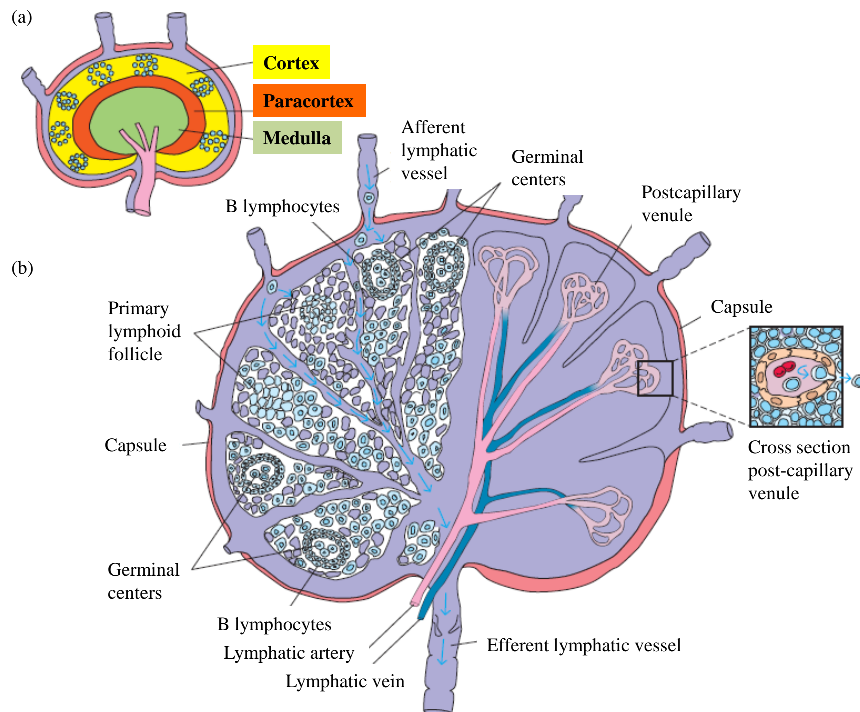


Figure 17. Structure of lymph nodes. (**a**) The three layers of a lymph node support distinct microenvironments. (**b**) The left side depicts the arrangement of reticulum and lymphocytes within the various regions of a lymph node (Goldsby *et al.,* 2003).

LNs are strategically located throughout the body (**Figure 18**) to allow lymphocytes to efficiently encounter their cognate antigen and become activated (Koning and Mebius, 2012). They are composed mostly of T-cells, B-cells, dendritic cells and macrophages, and nodes drain fluid from most tissues.

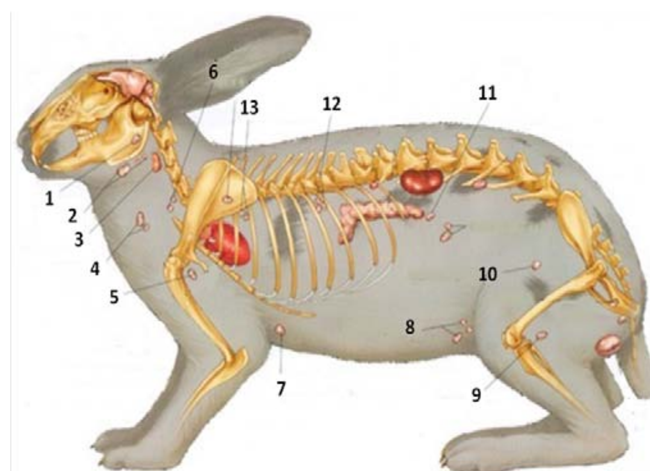


Figure 18. Main lymph nodes in rabbits: 1. LN parotid, 2. LN mandibular, 3. Thyroid gland, 4. Deep cervical LN, 5. Axilary LNs, 6. Superficial cervical LN, 7. Accessory Axilary LNs, 8. Epigastric LN, 9. Popliteal LN, 10. Inguinal LN, 11. Pancreatic LN, 12. Gastric LN, 13. Aortithoracic LN.

The functions of LNs are to filter lymphs from particulate matter and microorganisms, to facilitate the surveillance of incoming antigens and their interaction with B and T

lymphocytes, and to produce B lymphocytes (Fry and Donald McGavin, 2012). Antigens are filtered from the lymph into the lymph node before returning the lymph to circulation. Similarly to the spleen, the macrophages and dendritic cells that capture antigens present these foreign materials to T- and B-cells by consequently initiating an immune response.

Mucosa-Associated lymphoid tissues (MALT) are dispersed aggregates of non encapsulated organized lymphoid tissue within the mucosa (Elmore, 2006) situated on the surfaces of all mucosal tissue. The organization of MALT is similar to that of lymph nodes with B-cell-rich follicles and T-cell-rich interfollicular areas (Elmore, 2006). It is a site where an antigen is phagocytosed, and from which immune cells and antigen flow via afferent lymphatic vessels to regional lymph nodes (Fry and Donald McGavin, 2012).

Examples occur in the respiratory, gastrointestinal, urinary and reproductive tracts. Its most well-known representatives are gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT), and bronchus-associated lymphoid tissue (BALT); however, other associated lymphoid tissues have also been described (Cesta, 2006).

The primary function of MALT is to protect mucosal barriers. Thus, they are strategically placed to act as sentinels, but can also become a portal of entry for bacteria (i.e., Peyer´s patches) (Fry and Donald McGavin, 2012). Indeed, it plays a critical role as inductive sites for the initiation of antigen-specific protective immunity against the pathogens penetrating the mucus membrane (Okada *et al.,* 2011).

Another important secondary organ exclusive to rabbits is an anatomic structure located in the last part of the ileum, the **sacculus rotundus**, which appears to perform an immune system function. This intestinal structure contains a large amount of lymphoid tissue and its functions are similar to other GALT, which is sampling antigens from intestine contents (like food ingested by rabbits) and determining which antigens are the pathogens requiring an immune response (like harmful bacteria) and which are not harmful parts of food.

2.3. Cells of the immune system

Immune responses are mediated mainly by leukocytes and the soluble molecules which they secrete, although other tissue cells also participate. All the cells of the immune system derive from pluripotent bone marrow stem cells or HSCs, which gives rise to all the blood cell types from the erythroid lineage, and to myeloid and lymphoid lineages (**Figure 19**).

The erythroid lineage ends with the formation of erythrocytes and blood platelets. The myeloid cell line begins with a myeloblast. As myeloblasts reproduce and become more specialised or mature, they eventually develop into functional blood cells, including monocytes and macrophages, neutrophils, basophils, eosinophils and dendritic cells, which form the innate immune system.

Antigen-presenting cells (APCs) are a heterogeneous population of leukocytes that play an important role in innate immunity and that also act as a link to the adaptive immune system by participating in the activation of helper T-cells (Th-cells). These cells include dendritic cells and macrophages. A characteristic feature of APCs is the expression of a cell surface molecule encoded by genes in the major histocompatibility complex, referred to as class II MHC molecules. B lymphocytes also express Class II MHC molecules and they also

function as APCs, although they are not considered part of the innate immune system. In addition, some other cells (i.e., thymic epithelial cells) can express Class II MHC molecules and can function as APCs (Mayer and Nyland, 2006).

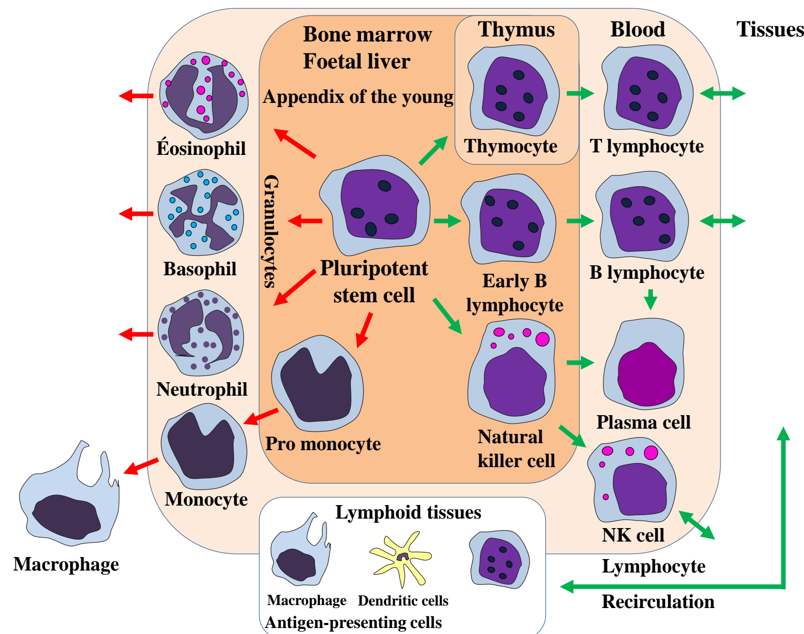


Figure 19. Origin of leukocytes. Differentiation from pluripotent stem cells (Adapted from Drouet-Viard and Fortun-Lamothe, 2002).

The lymphoid lineage generates natural killer cells, T-cells and B-cells, and the last two form the adaptive (specific) immune system.

2.3.1. Phagocytic cells

White blood cells or leukocytes are a diverse group of cell types that mediate the body's immune response. They circulate through the blood and lymphatic system, and are recruited to tissue damage and infection sites. Leukocyte subsets are distinguished by functional and physical characteristics. They have a common origin in hematopoietic stem cells, and they develop along distinct differentiation pathways in response to internal and external stimuli (Geissmann *et al.,* 2010).

When a pathogen attacks and the infection site reddens, the infected area swells and the temperature also rises. This kind of response is known as an inflammatory response. Such a response occurs due to the release of chemical alarm signals, such as histamine and prostaglandins, by damaged mast cells. Serum proteins are also released from blood vessels. Phagocytic cells, a subgroup of leukocytes, are also present in the affected area and represent one of the most important defence mechanisms in innate immunity (Mayer, 2006).

The acute inflammation process is initiated mainly by neutrophils, monocytes, tissue macrophages and dendritic cells. On their surfaces, these cells present pattern recognition receptors (PRRs), which include Toll-like receptors, NOD-like receptors, RIG-I-like receptors and C-type lectin receptors (Takeuchi and Akira, 2010). Moreover, they recognize molecules that are broadly shared by pathogens and are distinguishable from host molecules:

pathogen-associated molecular patterns (PAMPs). Effective sensing of pathogens PAMPs rapidly induces host immune responses via the activation of complex signaling pathways that culminate in the induction of inflammatory responses mediated by various mediators, which subsequently facilitate eradication of the pathogen (Medzhitov, 2007; Kumar *et al.,* 2009a; Blasius and Beutler, 2010; Kawai and Akira, 2010; Takeuchi and Akira, 2010) by directing the movement of circulating leukocytes to inflammation or injury sites (Charo and Randsohoff, 2006).

Phagocytic cells generally patrol the body while searching for pathogens, but they are also able to react to a group of highly specialized molecular signals produced by other cells called cytokines. They are recruited at infection sites during an inflammatory response where they perform a number of functions, such as ingesting and destroying pathogenic organisms, and neutralizing toxins (Snyder, 2012). Many receptors are involved in this process, some with signaling capabilities and some without. Increasing evidence reveals a previously unappreciated regulatory component to phagocytosis exerted by the concomitant engagement of signaling receptors. The engagement of Toll-like receptors (TLRs) during the phagocytosis of microbial pathogens is the best characterized example (Blander, 2008).

During phagocytosis, microorganisms or particles attach onto the phagocyte surface, the surface invaginates and the cell membrane completely surrounds the particle, or the pseudopodes move outward and surround the particle, forming a phagosome (Drouet-Viard and Fortun-Lamothe, 2002). In **Figure 20**, adapted from Kumar *et al.* (2009b), the intracellular destruction mechanism of microbes is simply represented.

As seen in **Figure 20**, following microbial internalisation, early phagosomes are subjected to numerous maturation steps that are concomitant with important changes in their associated proteins and luminal pH acidification. Acidification results from the fusion of phagosomes with pre-existing lysosomes, leading to the activation of hydrolytic enzymes which act optimally at an acidic pH (4.5–5.0) to enable the degradation of microorganisms (Henry *et al.,* 2004). In addition to acidification, another critical mechanism involves recruitment to the phagosomal membranes of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex subunits which, in turn, generate reactive superoxide ions to accumulate in the phagosomal lumen (Segal, 2005). Superoxide is further converted into several highly toxic radical oxygen species (ROS) that degrade phagosomal content and facilitate pathogen killing (Babior, 2000; Nathan and Ding, 2010). ROS generation also induces increased phagosomal pH which subsequently activates neutral proteases that digest and kill microorganisms. Upon activation, phagocytes generate other important antimicrobial effector molecules, such as the reactive nitrogen species (RNS) produced by inducible nitric oxid synthase (iNOS) that interacts with ROS to exert very toxic effects on intraphagosomal engulfed microorganisms (MacMicking *et al.,* 1997). Collectively, these antimicrobicidal effectors activities provide a toxic phagosomal environment that efficiently limits pathogen proliferation.

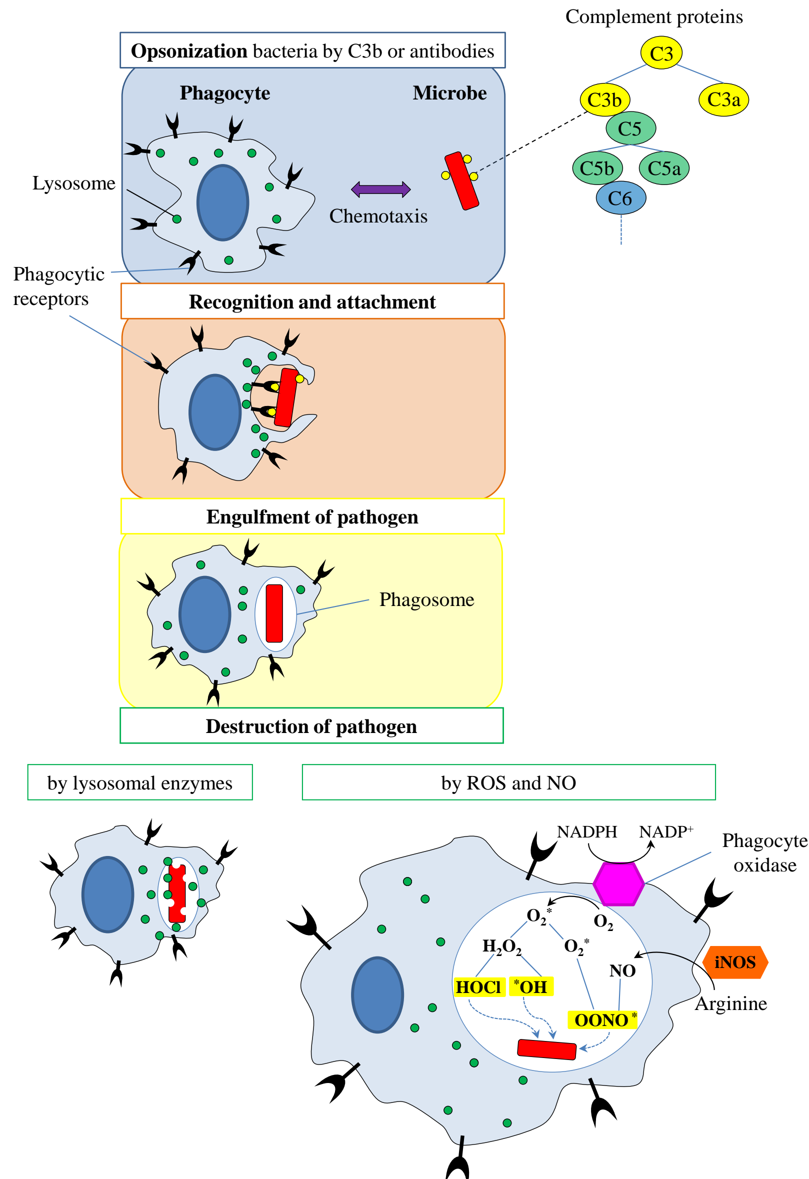


Figure 20. Phagocytosis and intracellular destruction of microbes. Phagocytosis of a particle (e.g., infectious microorganism) involves binding receptors on the leukocyte membrane, engulfment and fusion of lysosomes with phagocytic vacuoles. This process is followed by the destruction of ingested particles within the phagolysosomes by lysosomal enzymes, and by reactive oxygen and nitrogen species. The microbicidal products generated from superoxide are hypochlorite ($HOCl^*$) and hydroxyl radical ($^*OH$), and peroxynitrite ($OONO^*$) from nitric oxide (NO). During phagocytosis, granule contents may be released into extracellular tissues (not shown). *iNOS*: Inducible NO synthase; *ROS*: Reactive oxygen species (Adapted from Kumar *et al.,* 2009b).

2.3.1.1. Mononuclear phagocytes: monocytes and tissue macrophages

The term mononuclear phagocytic cells was developed to differentiate lymphoid cells, granulocytes and endothelial cells from lineage-committed precursors in BM, blood monocytes and tissue macrophages and dendritic cells that currently comprise the mononuclear phagocytic system (MPS). These cells perform a wide range of functions by contributing to immunity, inflammation, tissue remodeling and repair (Snyder, 2012).

In tissues, they are known as macrophages, but are called monocytes in blood. Monocytes and macrophages are critical effectors and regulators of inflammation; and the innate immune response is the immediate arm of the immune system (Geissmann *et al.,* 2010).

Monocytes are bone marrow-derived cells of the MPS of the myeloid linage that link inflammation and innate immune responses to adaptive immunity (Snyder, 2012). These cells migrate through blood vessels into various organs and tissue systems, where they become tissue macrophages (Roitt *et al.,* 1996).

The growth and differentiation of monocytes is regulated by specific growth factors, such as IL-3, colony-stimulating-factor-1 (CSF-1), granulocyte-macrophage CSF (GM-CSF), IL-4, and IL-13, and inhibitors such as type I interferons and transforming growth factor-β (TGF-β) (Snyder, 2012).

Two major functional subpopulations of monocytes are distinguished: one is recruited and differentiated into macrophages at the inflammation site and expresses higher levels of MHC Class II and adhesion molecules; the other is responsible for repopulating resident tissue macrophages. Both populations can give rise to dendritic cells (Snyder, 2012). These cells are very effective at presenting antigens to T lymphocytes. Their two main functions are to remove particular antigens by phagocytosis and to take up, process and present antigens to T-cells. Phagocytosis occurs when these cells bind microorganisms through specialized receptors for carbohydrates of the microbial cell wall, or to IgG, and to complement that which the microorganism has become coated with; the presentation of antigens to T-cells is mediated by MHC Class II. These functions can be enhanced by the ability to derive T-cell cytokines due to additional receptors which possess cytokines (IL-2, IL-4, IFNγ) and a migration inhibition factor. After activation complements components, prostaglandins and other cytokines are produced by them, including IFNs, IL-1 and TNFα (Roitt *et al.,* 1996).

Rabbit monocytes are the largest circulating leukocytes (15-18 µm in diameter). Monocytes have a large, variably-shaped nucleus with chromatin that appears less condensed than that of heterophils. The cytoplasm is abundant and a few cytoplasmic vacuoles are observed. Large, dark red granules have been described in the cytoplasm of some monocytes in association with non specific toxicity (Benson and Murphy, 1999). Ultrastructural monocytes possess many intracytoplasmatic lysosomes, which contain peroxidase and several acid hydrolases involved in the intracellular killing of microorganisms (Roitt *et al.,* 1996).

Tissue macrophages are professionally motile cells that carry out a variety of roles in immune surveillance and normal tissue development by secreting cytokines and growth factors, and by phagocytosing foreign material and apoptotic cells (Pixley, 2012), which also bridge innate and adaptive arms of immunity (Shapiro *et al.,* 2011).

Transendothelial and interstitial motility is an essential aspect of their function as they must be able to move to specific sites upon demand (Pixley, 2012). These leukocytes are strategically located throughout body tissues, where they ingest and process foreign materials, dead cells and debris, and recruit additional macrophages in response to inflammatory signals. These cells recognize danger signals through receptors capable of inducing specialized activation programs. The classically known macrophage activation is induced by IFNγ, synthesized by activated Th1 lymphocytes, which triggers a severe pro-

inflammatory response required to kill intracellular pathogens. Macrophages also undergo alternative activation by IL-4 and IL-13, which trigger a different phenotype which is important for the immune response to parasites (Martínez *et al.,* 2009).

Macrophages are highly heterogeneous cells whose function can rapidly change depending on the response to local micro-environmental signals (Murray and Wynn, 2011). Despite their heterogeneity, all macrophages originate from the pluripotent hematopoietic stem cell and, under the influence of hematopoietic growth factors, differentiate through several multipotent progenitor stages to lineage committed mononuclear phagocytic precursors in BM (Pixley and Stanley, 2004; Pollard, 2009). In accordance with their diverse activities, macrophages acquire differential phenotypes, dictated by the form, stage and site of lesion (Mosser and Edwards, 2008; Biswas and Mantovani, 2010). Macrophage polarization is broadly divided into two opposing phenotypes following lymphocyte terminology (Th1 and Th2 subsets), consisting of M1 and M2 macrophages (Mantovani *et al.,* 2004; Martínez *et al.,* 2009). Type 1 and type 2 inflammation represent ancient innate pathways with fundamentally different purposes. Type 1 promotes the killing of microbial pathogens and intracellular parasites, and is involved in tissue destruction and tumor resistance. Type 2 participates in tissue repair and controls infection with macroparasites through encapsulation (Willem and Fieren, 2012). As a result, macrophages initially down-regulate their pro-inflammatory activity and shift their function to the local resolution of inflammation and tissue repair (Shapiro *et al.,* 2011). Additionally, macrophages express Fc receptors (FcR) for an antibody and can phagocytose antigens opsonized by an antibody or complement components. Additionally, they express MHC Class II molecules upon activation and are regarded as major antigen-presenting cells by phagocytozing antigens and by processing them into peptide fragments, which are then presented to T lymphocytes and the induction of cell-mediated immune responses (Snyder, 2012).

2.3.1.2. Dendritic cells

Dendritic cells (DCs) are the phagocytic cells mainly present in tissues and in contact with the external environment, the skin (where they are called Langerhans cells), and in the inner mucosal lining of the nose, lungs, stomach and intestines (Bruce *et al.,* 2002). In addition, circulating and follicular dendritic cells exist, which are located in peripheral blood or lymphoid organs, respectively (Snyder, 2012). Upon stimulation, dendritic cells transform to a mature phenotype characterized by high levels of the class II major histocompatibility complex (MHC class II) and co-stimulatory molecules with poor phagocytic capacity (Kinsey and Okusa, 2012).

Mature dendritic cells are characterized by elongated cell processes and a number of surface molecules, such as TLRs and mannose receptors, which permit them to capture antigens and to migrate via afferent lymphatic vessels into lymph nodes. In lymph nodes, DCs carry antigens from the skin to the T lymphocyte areas of secondary lymphoid organs where they present fragments of antigens on their surface and increase their expression of co-stimulatory molecules, which activates T lymphocytes (Snyder, 2012).

Dendritic cells initiate and regulate highly pathogen-specific adaptive immune responses, and are central to the development of immunologic memory and tolerance (Geissmann *et al.,* 2010). In particular, most dendritic cells are very important for the antigen presentation

process (Snyder, 2012) and in the innate immune because they are the responses through several mechanisms (Kinsey and Okusa, 2012). There are four types of dendritic cells:

- Follicular - which promotes the differentiation of B-cells.
- Myeloid - which stimulates Th1 cells.
- Lymphoid - which activates Th2 cells.
- Plasmacytoid - which links the innate and adaptive immune responses upon maturation.

2.3.1.3. Polymorphonuclear granulocytes

There is a group of cells that presents a polymorphic nucleus and can be distinguished into neutrophils, basophils and eosinophils.

Neutrophils are phagocytic cells that represent the majority of blood leukocytes and are produced in BM from stem cells that proliferate and differentiate to mature neutrophils, which are fully equipped with an armory of granules. These contain proteins that enable the neutrophil to deliver lethal actions against microorganisms, but also cause great tissue damage. Neutrophils circulate in the blood as dormant cells (Borregaard, 2010). In rabbits, heterophils perform the same function as other mammalian neutrophils, but their citoplasm contains acidophilic or eosinophilic granules, which is why they are named heterophils in this animal species. Their size ranges from 10 to 15 µm in diameter. Polymorphonuclear neutrophils (PMNs) have a light purple, lobulated nucleus surrounded by the cytoplasm containing diffuse, variably-sized reddish granules. Heterophilic granules are generally smaller than those of eosinophils and may not occupy all the cytoplasm. The functions of PMNs in rabbits are similar to those of other mammals (Lester *et al.,* 2005). These cells play an important role in innate immunity by defending the host against invading microorganisms (Saffarzadeh *et al.,* 2012). A critical aspect is that neutrophils must be activated for host defense. Activation of neutrophils can be induced by various stimuli, including pathogen-derived molecules and several chemo-attractants (Walther *et al.,* 2000; Tiffany *et al.,* 2001; Sabroe *et al.,* 2003; Kobayashi, 2008), including several chemokines that regulate the activities of neutrophils.

Neutrophils are recruited to infected areas or injury sites according to a chemo-attractant gradient (Borregaard, 2010; Kumar and Sharma, 2010). After recruitment, neutrophils specifically recognize invading pathogens prior to their removal through its membrane receptors, and subsequently attack invading pathogens by releasing antimicrobial peptides and lytic enzymes, and producing reactive oxygen species (ROS) followed by phagocytosis, which enables the clearance of invading pathogens (Decoursey and Ligeti, 2005; Nathan, 2006). Phagocytosis of bacteria typically accelerates neutrophil apoptosis, which ultimately promotes the resolution of infection (Kennedy and DeLeo, 2009). Another recently described antimicrobial mechanism of neutrophils is the formation of neutrophil extracellular traps (NETs), stands of DNA with bactericidal proteins attached which act as extracellular traps for microorganisms. As PMNs are the most abundant type of leukocytes and contain an arsenal of cytotoxic compounds that are non specific, neutrophil homeostasis must be highly regulated. Constitutive PMNs turnover is regulated by apoptosis, a process whereby these cells shut down and are removed safely by macrophages (Kobayashi *et al.,* 2005). This mechanism is central to the successful resolution of an inflammatory response and it is increasingly apparent that the dying neutrophil itself exerts an anti-inflammatory effect

through the modulation of surrounding cell responses, particularly macrophage inflammatory cytokine release (Fox *et al.,* 2010).

Basophils and **eosinophils** are cells related to the neutrophil, but they are not phagocytic cells. In rabbits, basophils have a light purple, lobulated nucleus, and dark purple to purple-black cytoplasmic granules. Basophils are approximately the same size as heterophils, while eosinophils are slightly larger than heterophils (12 to 16 μm in diameter). The nucleus stains purple and often appears bilobed. Intensely acidophilic, round, cytoplasmic granules are present, which are larger and more numerous than the granules in heterophils (Lester *et al.,* 2005). Eosinophils appear to be capable of phagocytosing and killing ingested microorganisms, although they play a specialized role in immunity to parasites by releasing granule contents into the surrounding area (Roitt *et al.,* 1996), including a range of highly toxic proteins and free radicals that are highly effective in killing bacteria and parasites, but are responsible for tissue damage during allergic reactions (Stvrtinová *et al.,* 1995). When activated by a pathogen encounter, the basophils releasing histamine are important in defense against parasites and play a role in allergic reactions (Roitt *et al.,* 1996).

2.3.2. Mast cells

Mast cells (MCs) are a type of innate immune cell that resides on the interface between the host and the external environment near blood vessels, lymphatic vessels, nerve fibers and a range of immune cells, including dendritic cells (Kunder *et al.,* 2009). This strategic positioning allows them to act as sentinels of invading microbes, but to also respond rapidly to any change in the environment by communicating with the different cells involved in both physiological and immunological responses (Weller *et al.,* 2011). MCs are intimately associated with defense against pathogens and wound healing, but are also associated frequently with allergy and anaphylaxis (Kunder *et al.,* 2011). Recent findings support the possibility of MCs playing an important sentinel and effector role during bacterial infection that helps promote the clearance of pathogens by phagocytosis and/or the secretion of anti-microbial peptides, and protects hosts from pathology and enhances survival (Galli and Tsai, 2010). In particular, a number of studies have confirmed that MCs play a critical role in host immune defense against Gram-negative bacteria through the release of TNFα, leukotrienes, tryptase, etc. (Malaviya, 2008).

MCs express receptors on the surface, which are able to “sense” pathogens (Galli and Tsai, 2010). Their activation induces the release of the preformed inflammatory mediators localized in specialized granules (histamine and heparin), as well as the *de novo* synthesis and secretion of cytokines, chemokines (such as TNF, IL-4, IL-5, IL-6 and IL-13), and eicosanoids (leukotrienes and prostaglandins) (Rivera and Gilfillan, 2006), which contribute to protective immunity and pathogen clearance or containment (Kunder *et al.,* 2009; Galli and Tsai, 2010). Kumar *et al.* (2009b) represent this phenomenon simply in the **Figure 21**.

The plethora of mediators and the speed at which some of these mediators are released from MCs make the control of MC activation a key element to their functions (Weller *et al.,* 2011). Histamine dilates blood vessels by causing characteristic signs of inflammation, and recruits neutrophils and macrophages (Lippincott and Wilkins, 2007).

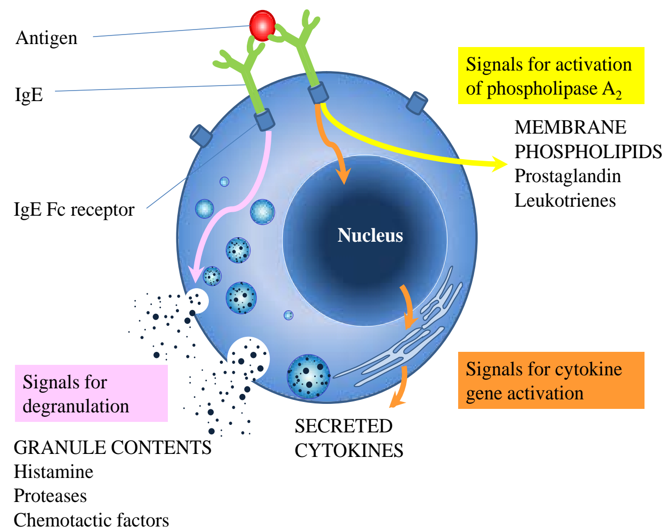


Figure 21. Mast cell degranulation and activation (Adapted from Kumar *et al.,* 2009b).

2.3.3. Natural killer cells

Natural killer (NK) cells play a multitude of roles in host innate and adaptive immunity (Moretta *et al.,* 2008), apart from a newly recognized immunological memory capacity (Megan *et al.,* 2009). They were named “natural killers” based on the initial notion that they did not require activation to form their cytotoxic functions. NK cells are widely distributed in the body and their number may increase locally during infection. They develop mainly in BM, and perhaps in other lymphoid organs. They are constantly renewed (Rouzaire *et al.,* 2012). It is well-known that NK cells derive from $CD34^+$ hematopoietic progenitor cells (HPCs) and that IL-15 is crucial for their development and homeostasis. Acquisition of the β-chain of the IL-2/15 receptor (CD122) marks one of the earliest steps of NK cell commitment (Mujaj *et al.,* 2011). These NK precursors (NKPs) lack the expression of T-cell associated proteins and are unable to differentiate into B- , T- , myeloid and erythroid cells, but can be stimulated to form mature NK cells *in vitro* (Rosmaraki *et al.,* 2001). As NKPs mature, they gradually acquire more of the receptors seen in mature NK cells. As these cells mature further, their proliferation rate slows and an increased CD43 expression, cytotoxicity and IFN-γ production distinguish mature NK cells (Kim *et al.,* 2002a; Boos *et al.,* 2008). Acquisition of the high-affinity β-chain of the IL-15 receptor, CD122, is an essential step in the commitment of HPCs to the NK cell lineage in both humans and mice. Therefore, IL-15 is essential for the development of mature NK cells, while the generation of NKPs from HPCs is IL-15-independent (Kennedy *et al.,* 2000; Vosshenrich *et al.,* 2005).

They possess a morphology of large granular lymphocytes and their receptor genes, which are involved in the recognition of pathogens, remain in a germinal configuration, unlike T- and B-cells. They also differ from them given the permanent presence of a significant fraction of educated, primed cells (Langers *et al.,* 2012). These cells enter regions of acute inflammation, hours and even days after lesion initiation (Ackermann, 2012), and they are able to detect and lyse cells with a deficient expression of major histocompatibility complex class I (MHC-I) molecules (Langers *et al.,* 2012). This scenario permits them to recognize and lyse tumor- and pathogen-infected cells with no prior stimulation (**Figure 22**). This is achieved via direct lysis, and indirectly by antibody-dependent cellular cytotoxicity (Moretta *et al.,* 2008). NK cells kill target cells through the release of perforin from cytoplasmatic

granules (Ackermann, 2012). Another function performed by these cells is to promote B- and T-cell differentiation and dendritic cell (DC) maturation (Moretta *et al.,* 2008).

They express CD161, a C-type lectin, but do not express CD3, the T lymphocyte antigen, and roughly 95% of them express CD56 and produce IFN-γ (Ackermann, 2012), which enhance the innate immune response and help shape the subsequent adaptive immune response (Strowig *et al.,* 2008); these are type I NK cells. Type II NK cells lack the CD56 expression and produce IL-4, IL-5, and IL-13, thus supporting a Th2 response (Ackermann, 2012).

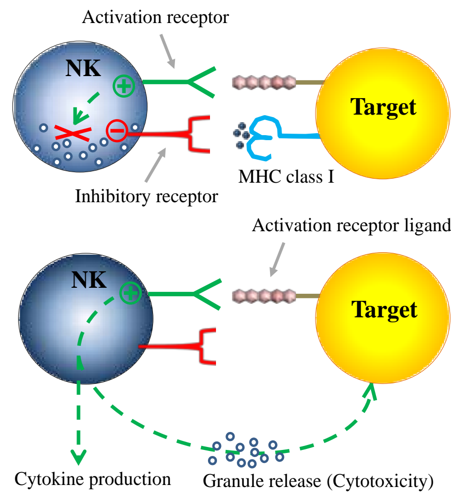


Figure 22. Natural killer (NK) cell activation is controlled by the integration of signals from activation and inhibitory receptors. (**a**) Inhibitory NK cell receptors recognize self MHC Class I and restrain NK cell activation. (**b**) When unimpeded by inhibitory receptors, the binding of NK cell activation receptors to their ligands on target cells results in NK cell stimulation. In the absence or with the down-regulation of self MHC Class I on target cells, these stimulatory signals are no longer suppressed, resulting in NK cell responses, including cytokine production and granule release, which lead to cytotoxicity. Note that this model indicates that NK cells do not kill by default; that is, when MHC Class I inhibition is absent, the NK cell must still be stimulated through activation receptors. Moreover, whether or not an individual NK cell is activated by a target is determined by this complex balance of receptors with an opposing function and expression of the corresponding ligands. However, inhibition dominates activation, in general. Finally, NK cells can be directly stimulated by cytokines, such as interleukin-12, which trigger the production of other cytokines by NK cells. These direct cytokine-mediated responses are not affected by the MHC Class I expression (Adapted from French and Yokoyama, 2004).

Unlike adaptive T and B lymphocytes, NK cells do not rearrange their receptor genes somatically, but rely on a fixed number of inhibitory and activating NK cell receptors (NKRs) capable of recognizing MHC Class I and Class I-like molecules, as well as other ligands (Bryceson and Long, 2008). Tolerance of NK cells to self is achieved through mechanisms that require the engagement of inhibitory NKRs with self-MHC before attaining functional competence, a process termed 'licensing' (Kim *et al.,* 2005; Raulet and Vance, 2006; Jonsson and Yokoyama, 2009). NK cell effector functions can be triggered by the engagement of activating NKRs with cell-surface ligands, which can be encoded by the host or by pathogens, some of which are up-regulated in infected cells or tumors (Arase *et al.,* 2002; Smith *et al.,* 2002; Guerra *et al.,* 2008). NK cells also respond to other signals,

especially the cytokines deriving from antigen-presenting cells, which allows them to mediate early host responses against pathogens (Andrews *et al.,* 2003; Moretta *et al.,* 2006).

Traditionally, human NK cells are identified by the expressions of CD16 and CD56, and mouse NK cells by the NK1.1 marker (designated as CD161).

2.3.4. Lymphocytes

Lymphocytes derive from bone-marrow stem cells (Roitt *et al.,* 1996). Yet it has been suggested in recent studies performed in mouse embryos that T lymphocytes derive directly from the hemogenic endothelium before hematopoietic stem cells appear (Yoshimoto *et al.,* 2012).

These leucocytes are responsible for initiating the adaptive immune system response (Roitt *et al.,* 1996). However, recent studies report that T lymphocytes may also be involved in innate immune responses (Kabelitz, 2007; Oberg *et al.,* 2011). In fact there are basically two different types of lymphocytes: T lymphocytes (T-cells) that are involved in cell-mediated immunity and B lymphocytes (B-cells) that are involved in humoral immunity (Snyder, 2012). Another small number of circulating hematopoietic stem cells exists, which are natural killer (NK) cells, resembling lymphocytes.

Lymphocytes are a heterogeneous collection of cells that can be easily distinguished from other leukocytes by their characteristic morphology. Naïve lymphocytes, cells which were not previously stimulated by antigens, are called small lymphocytes by morphologists. In circulating blood, lymphocytes may be either small lymphocytes (6-9 µm) or large lymphocytes (9-15 µm) (Ford and Gowans, 1969). They have a large nucleus with a dense heterochromatin and a thin cytoplasm rim that contains a few mitochondria, ribosomes and lysosomes, but no specialized organelles (**Figure 23**).

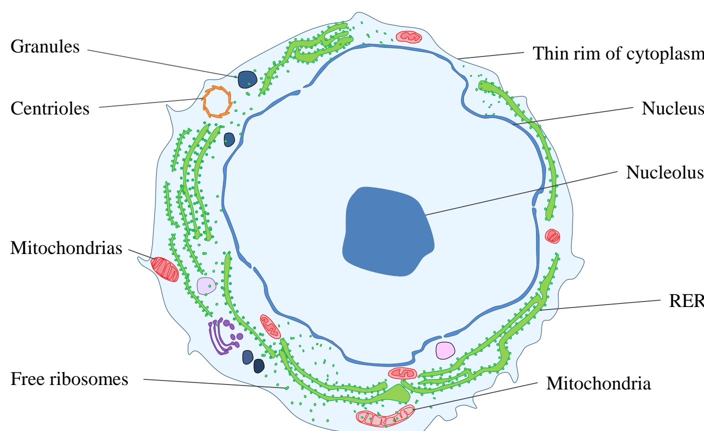


Figure 23. Morphology of lymphocytes. *RER*: Rough endoplasmic reticulum.

In the absence of antigenic stimulation, virgin mature lymphocytes die by apoptosis within a few days, while an interaction of small lymphocytes with antigens, in the presence of certain cytokines, induces these cells to enter the cell cycle by progressing from G_0 into G_1, and subsequently into S, G_2, and M (**Figure 24**). As they progress through the cell cycle, lymphocytes enlarge into blast cells, called lymphoblasts. These cells have a higher cytoplasm:nucleus ratio and greater organellar complexity than small lymphocytes.

Lymphoblasts proliferate and eventually differentiate into either effector cells or memory cells. Effector cells function in various ways to eliminate antigens. These cells have short life spans, generally ranging from a few days to a few weeks (Goldsby *et al.,* 2003).

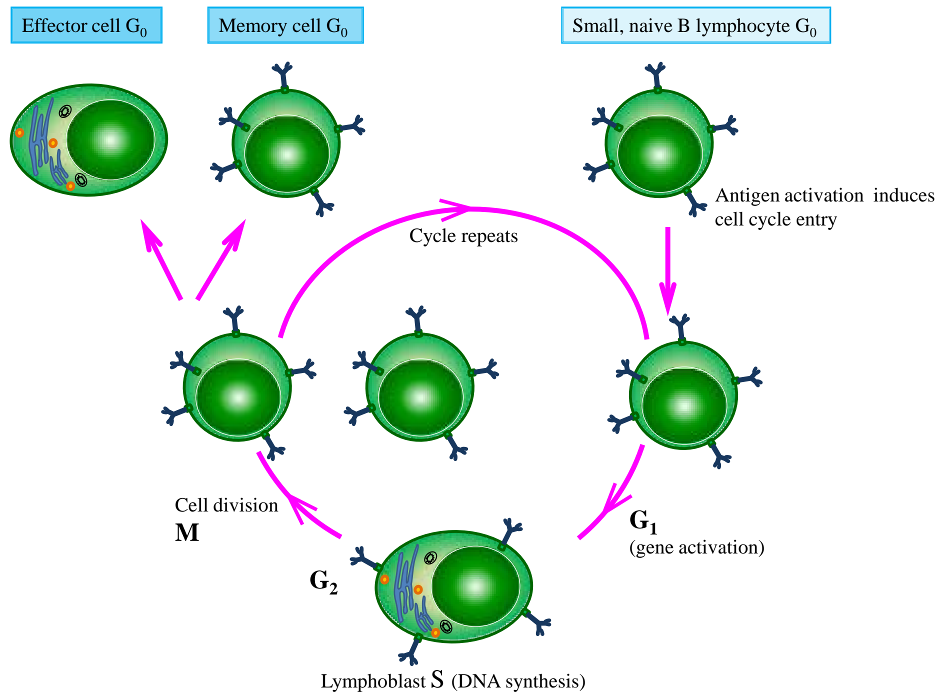


Figure 24. Fate of small antigen-activated lymphocytes. A small resting (naïve or unprimed) lymphocyte resides in the G_0 phase of the cell cycle. At this stage, B and T lymphocytes cannot be distinguished morphologically. After antigen activation, a B- or T-cell enters the cell cycle and enlarges into a lymphoblast, which undergoes several rounds of cell division, and, eventually generates effector cells and memory cells. Here cells of the B-cell lineage are shown (Adapted from Goldsby *et al.,* 2003).

The common lymphoid progenitor is capable of differentiating into either B- or T-cells, depending on the primary lymphoid organ elected for the following maturation. Moreover, lymphocyte development requires the concerted action of a network of cytokines and transcription factors, which positively and negatively regulate gene expression (LeBien and Tedder, 2008). In primary lymphoid organs, lymphocytes develop specific surface receptors permitting them to cope with antigenic challenges received during their lifespans (Roitt *et al.,* 1996). Once they have migrated to the peripheral lymphoid tissue, they may undergo clonal expansion in response to antigenic stimulation (Snyder, 2012). Abbas and Lichtman (2005) simply represent the maturation of lymphocytes in the lymphoid organs in **Figure 25**.

Mature lymphocytes cells are strategically located in areas that come in close contact with foreign substances, such as the small intestine. They represent one of the first lines of defense against invading microorganisms, viruses and parasites. In such locations, they are perfectly positioned to interact with invading foreign substances and they recognize these substances as non-self or foreign. Upon such "recognition", lymphocytes are activated and function to neutralize or destroy the invading foreign substance.

Free lymphocytes can be found in the epithelium and the lamina propria of the tunica mucosa of digestive, respiratory, urinary and reproductive tracts, which is a good location for

detecting foreign substances. As with macrophages, lymphocytes enter unresolved areas of acute inflammation within 24-48 hours, and are attracted by chemokines, cytokines and other stimuli. Histologically, they are often aggregated around blood vessels and they surround injured tissue (Mosier, 2012).

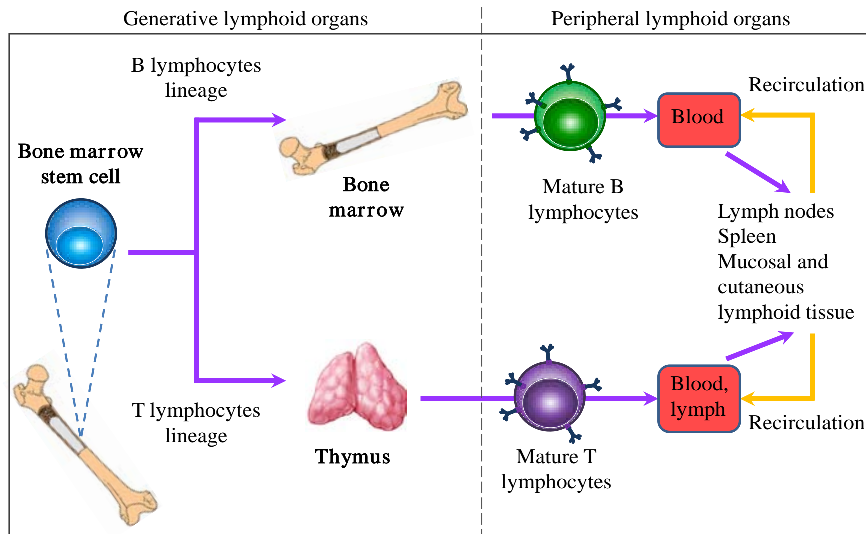


Figure 25. Maturation of lymphocytes. Mature lymphocytes develop from bone marrow stem cells in generative (primary) lymphoid organs, and immune responses to foreign antigens occur in peripheral (secondary) lymphoid tissues (Adapted from Abbas and Lichtman, 2005).

It has been reported that the morphology of rabbit lymphocytes is similar to that of other species. Lymphocytes have a large nucleus, which may be slightly indented, and can have a small amount of light blue cytoplasm. Although small lymphocytes predominate, large lymphocytes may be present. These cells are similar to heterophils in size. Large lymphocytes may occasionally contain azurophilic granules near the nuclear indentation. Reactive lymphocytes (immunocytes) are antigenically-stimulated lymphocytes and are larger cells with a more intensely blue cytoplasm (Lester *et al.,* 2005).

2.3.4.1. B lymphocytes

B lymphocytes are a subpopulation of leukocytes that express clonally diverse cell surface immunoglobulin (Ig) receptors by recognizing specific antigenic epitopes (LeBien and Tedder, 2008). B-cells are an essential component of the adaptive immune system and play a key role in the humoral immune response, whose principal function is to synthesize antibodies against soluble antigens.

The discovery and characterization of B-cells occurred in the mid-1960s and early 1970s using experimental animal models, clinical evaluation of patients with immune deficiency diseases (Good and Zak, 1956), and by applying the nascent technology of cell surface molecule characterization (LeBien and Tedder, 2008). The abbreviation "B" comes from bone marrow or from bursa of Fabricius, which is an organ in birds in which avian B-cells mature.

B lymphocytes can be found in primary lymphoid tissues, such as fetal liver, BM and ileal Peyer´s patches, and in secondary lymphoid tissues, such as spleen, lymph nodes, tonsils,

and Peyer´s patches (Snyder, 2012). Only a few circulate through blood (Tizard, 2009), moving to various sites throughout the body.

Recent studies have shown that naive B lymphocytes constitute the majority of the total B-cell population in non lymphoid tissues and suggest that these cells may re-circulate through the periphery as part of their normal migration pathway (Inman *et al.,* 2012).

B lymphocytes development occurs in two phases: an antigen-independent phase in primary lymphoid tissues; an antigen-dependent phase in secondary lymphoid tissues (**Figure 26**).

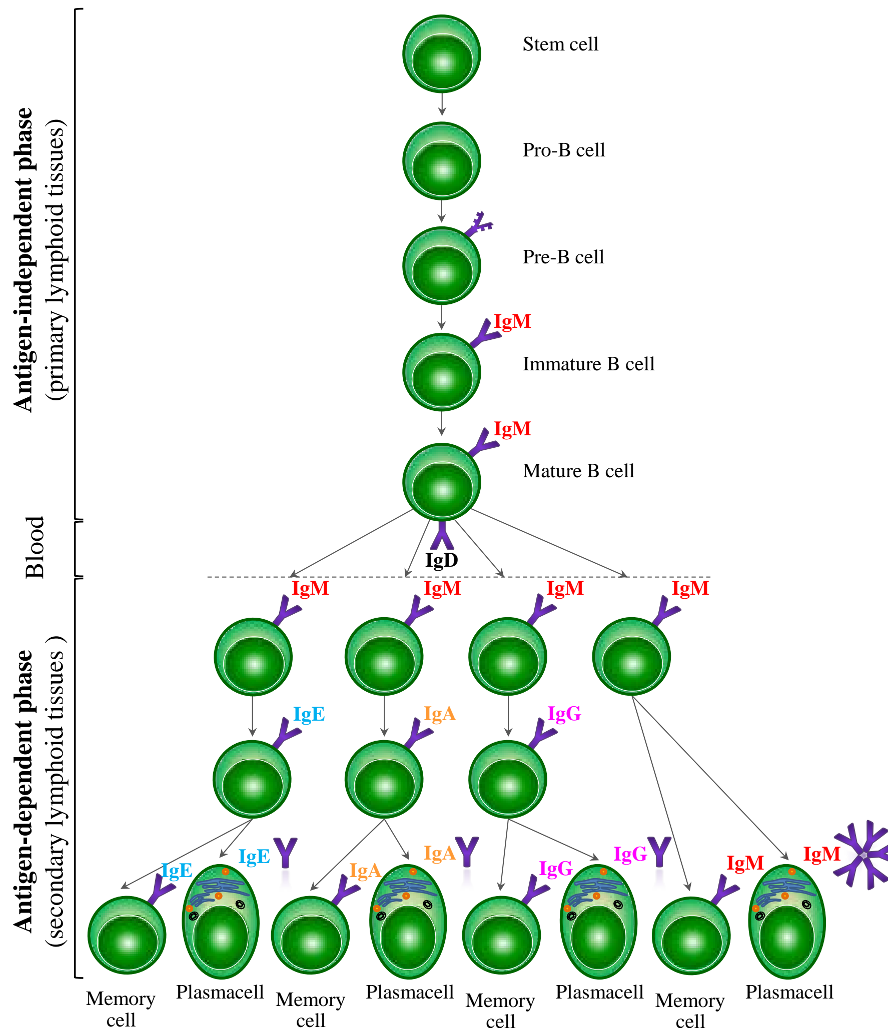


Figure 26. Maturation and clonal selection of B lymphocytes.

The antigen-independent phase

The process of B-cells maturation from undifferentiated precursors takes place in the fetal liver and after birth in BM, where the differentiation of hematopoietic progenitor cells in immunocompetent mature B-cells involves a sequence of rearrangements of immunoglobulin genes in the absence of foreign antigens. However, subsequent functional maturation occurs in secondary lymphoid tissues.

An antibody is composed of two light (L) and two heavy (H) chains, and the genes specifying them are found in both the 'H' chain and the 'L' chain loci. In H chain loci, there are three regions (V, D and J) which recombine randomly during a process called VDJ recombination to produce a unique variable domain in the immunoglobulin of each individual B-cell (**Figure 27**).

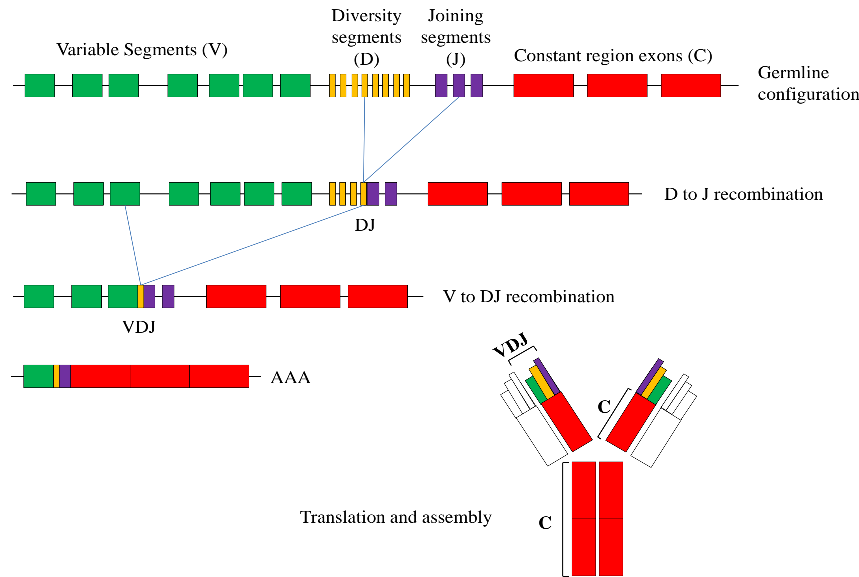


Figure 27. The V(D)J recombination.

Similar rearrangements occur for L chain loci, except there are only two regions, namely V and J. This B-cell development is characterized by the various maturation stages (**Table 2**).

Table 2. The development of a B-lineage cell proceeds through several stages marked by the rearrangement and expression of immunoglobulin genes. *D*: Diversity genes; *J*: Joining genes; *V*: Variable genes.

	Stem cell	Early pro-B cell	Late pro-B cell	Large pre-B cell	Small pre-B cell	Immature B-cell	Mature B-cell
H-chain genes	Germline	D-J rearranging	V-DJ rearranging	VDJ rearranged	VDJ rearranged	VDJ rearranged	VDJ rearranged
L-chain genes	Germline	Germline	Germline	Germline	V-J rearranging	VJ rearranged	VJ rearranged

The antigen-dependent phase

After reaching the IgM^+ immature stage in BM, mature immunocompetent B lymphocytes, called "virgin" or "naïve", migrate to the spleen where they mature into B lymphocytes. Subsequently through a gene rearrangement process, these lymphocytes specialize in the production of a single class of immunoglobulins IgG, IgA, IgM, IgD and IgE.

If a B-cell interacts with an antigen for which the antibody is a specific-membrane, the cell begins clonal expansion and differentiation, which result in a population of memory B-cells and plasma cells (**Figure 28**).

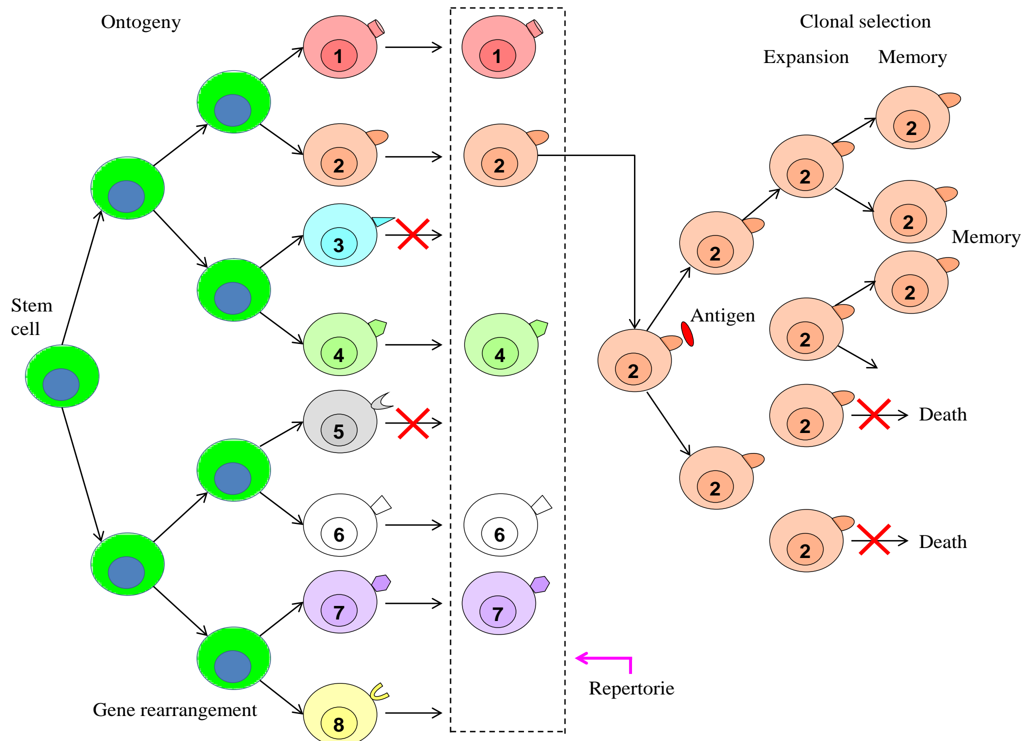


Figure 28. Clonal selection and expansion of the vertebrate immune response. After the exposure to an antigen, the immune system generates a repertoire of immune cell lineages or clones (labelled 1-8 in the above Figure), each encoding a receptor with a predetermined shape and specificity. The human immune system creates different clones in excess. As a first approach, those that react with self-antigens (numbers 3, 5, and 8) are deleted shortly after they mature. When the individual is infected with a pathogen, the clones that are specific for the pathogen (number 2) proliferate, producing a pathogen-specific immune cell population that is large enough to control that pathogen. This process is known as clonal expansion. After the pathogen is cleared, some of the pathogen-specific immune cells survive and confer immune memory (Bergstrom and Antia, 2006).

When the B-cell fails in any of the maturation process steps (i.e., when it recognizes self-antigens during the maturation process, the B-cell becomes suppressed (known as anergy) or undergoes apoptosis (a process called negative selection) (**Figure 29**).

Memory B-cells perform the same role for antibody-mediated immunity as memory T-cells perform for cell-mediated immunity. Memory B-cells do not respond to threat upon first exposure, but remain in reserve over long periods to deal with subsequent injuries or infections that involve the same antigens (Roitt *et al.,* 1996).

Plasma cells are terminally differentiated, non dividing, effector cells of the B-cell lineage (McHeyzer-Williams and McHeyzer-Williams, 2005; Shapiro-Shelef and Calame, 2005; Radbruch *et al.,* 2006; Fairfax *et al.,* 2008) and are rarely found in circulation, but reside in secondary lymphoid organs and at immune response sites. They form, mucosal surfaces and wound sites (Akermann, 2012) within lymph nodes and die by apoptosis after few days. They are the primary mediators of humoral immunity and secrete major classes of immunoglobulins: IgM, IgD, IgG, IgA and IgE (**Figure 30**).

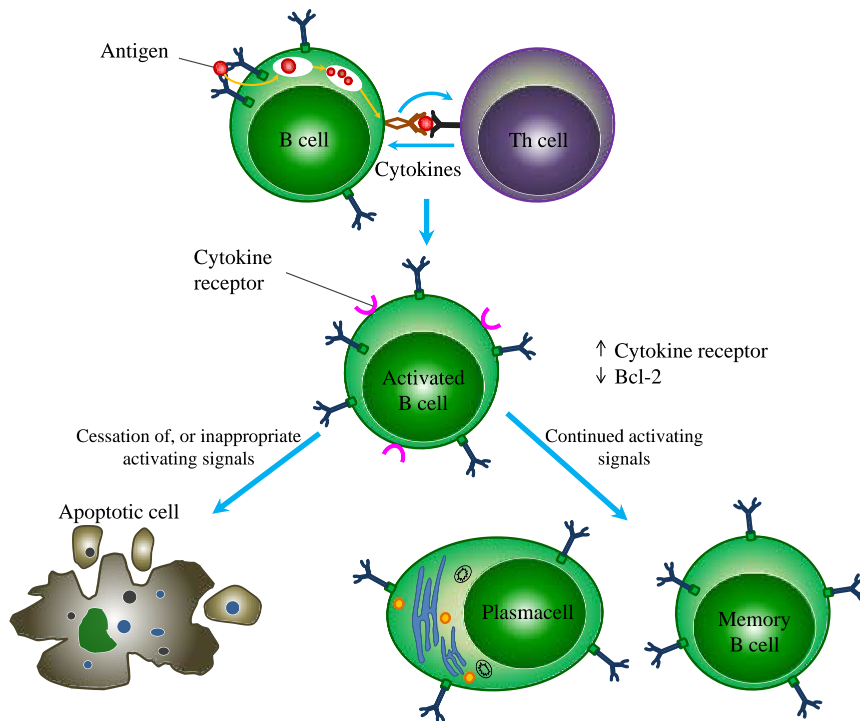


Figure 29. Regulation of activated B-cell numbers by apoptosis. The activation of B-cells induces an increased expression of cytokine receptors and a decreased expression of Bcl-2. Since Bcl-2 prevents apoptosis, its reduced level in activated B-cells is an important factor in making activated B-cells more susceptible to programmed cell death than either naïve or memory B-cells. A reduction in activating signals quickly leads to the destruction of excess activated B-cells by apoptosis. *Bcl-2*: B-cell lymphoma-2 (Adapted from Goldsby *et al.,* 2003).

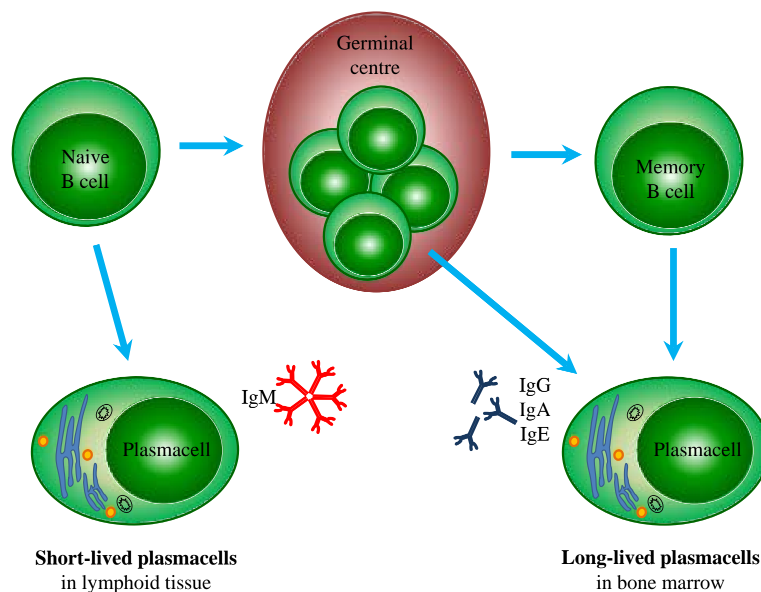


Figure 30. Development of memory B-cells and effector B-cells (plasma cells) occurs in two phases. Short-lived plasma cells that make mostly IgM (but some IgG) are generated during the primary response and occupy sites, such as lymph nodes. The second phase involves the formation of the memory B-cell pool and the seeding of long-lived plasma cells in bone marrow. Plasma cells do not give rise to memory cells. All the arrows are driven by antigen and T-cell help. *Ig*: Immunoglobulin (Gray, 2002).

Whereas many studies on peripheral B-cell development have been performed in mice and humans, few studies conducted in rabbits or other mammals that use GALT for B-cell expansion and the somatic diversification of Ig genes are available (Weinstein *et al.,* 1994b; Parng *et al.,* 1996; Vajdy *et al.,* 1998; Butler *et al.,* 2000; Weill and Reynaud, 2005; Meyer-Bahlburg *et al.,* 2008). In rabbits, B-cells leave BM and migrate to GALT, where they expand in numbers and somatically diversify the Ig genes (Becker and Knight, 1990; Weinstein *et al.,* 1994a, b; Knight and Winstead, 1997), while the diversification of the repertoire uses the gene conversion mechanism.

B-cell development occurs primarily through the classical, or ordered, pathway and shows that B lymphopoiesis reduces by over 99% by 16 weeks of age (Jasper *et al.,* 2003), which is probably due to the decline in the periostin expression in adult rabbit BM, which explains the arrest of B-lymphopoiesis (Siewe *et al.,* 2011). However, the interaction of periostin, a secreted extracellular matrix protein, with αvβ3 integrin in lymphoid progenitors, probably provides both proliferative and survival signals for cells in the B-cell development pathway (Siewe *et al.,* 2011). In adult rabbits, B lymphopoiesis is suppressed at a lymphoid progenitor stage (MHCII-IL-7 binding) of development (Kalis *et al.,* 2007). Therefore, rabbits may have evolved a strategy to rapidly differentiate their immature B-cells and to generate a functional B-cell compartment before the arrest of B lymphopoiesis (Yeramilli and Knight, 2011). GALT is characteristically composed of many follicles, which provide the microenvironment within which the diversification of the rabbit primary antibody repertoire occurs (Vajdy *et al.,* 1998; Lanning *et al.,* 2000b; Mage *et al.,* 2006). Follicles with germinal centers in the appendix (*processus vermiformis*), one of the largest organized GALTs, can be detected by week 2 post-birth (Thorbecke, 1960; Barg and Draper, 1977; Barg *et al.,* 1978; Weinstein *et al.,* 1994a, b). Rabbits lacking GALT have low serum Ig levels, fewer numbers of circulating lymphocytes, and diminished antibody responsiveness to several antigens (Cooper *et al.,* 1968; Dasso and Howell, 1997).

The B-cells outside marrow are morphologically homogenous, but their cell surface phenotypes, anatomic localization and functional properties reveal still-unfolding complexities. Immature B-cells are also referred to as “transitional” (T1 and T2), based on their phenotypes and ontogeny (Yeramilli and Knight, 2011). Transitional B-cells and their maturation have been studied mostly in mice (Vossenkämper and Spencer, 2011).

Transitional B-cells are identified by several cell surface markers expressed on newly formed B-cells in BM, and they mark a crucial link between immature BM B-cells and mature peripheral B-cells. These cells have a diversified repertoire and undergo proliferation. A subpopulation of B-cells is maintained by self-renewal and is responsible for maintaining the B-cell compartment in the absence of detectable B lymphopoiesis.

The majority of mature B-cells outside GALT reside within lymphoid follicles of spleen and lymph nodes, where they encounter and respond to T-cell-dependent foreign antigens bound to follicular dendritic cells, proliferate (Yeramilli and Knight, 2011), and then either differentiate into IgM-secreting plasma cells or switch to another antibody isotype (Snyder, 2012). Upon entry into the periphery, mature B-cells are ready to respond to their cognate antigen which leads to the activation of B-cells and antibody production. This process involves a large number of B-cells components, especially mIg on the surface, as well as the following: the associated structures forming the B-cells’ antigen receptor (BCR); B-cells’ co-

receptor complex induced in signal transduction; B-cells' activation in response to antigenic stimuli.

There are two subpopulations of B-cells:

- ✓ Lymphocytes B1 have been identified in humans, guinea pigs, mice, pigs, rabbits, sheep and cattle. They originate from stem cells in the fetal liver or omentum, but not in BM. Furthermore, two groups, B1a, which are held only in neonatal animals, are capable of self-renewal and are responsible for immunoglobulin M production. These lymphocytes recognize common bacterial molecules and antibodies in a T-independent manner. Adhesion molecules express CD5. Moreover, the B1b subpopulation differs from previous ones lacking CD5 and is produced during adulthood, and their main function is to defend against parasites and bacteria (Tizard, 2009).
- ✓ Lymphocytes B2 are conventional cells whose main function is humoral immune response development (Tizard, 2009).

2.3.4.2. T lymphocytes

Hematopoietic progenitors of myeloid, lymphoid or mixed myeloid/lymphoid potential enter the thymus and, although a fraction of these differentiate into dendritic cells, NK cells, B-cells or γδ T-cells, the majority yield αβ T-cells (Chi *et al.,* 2009), in which they gradually acquire T-cell characteristics while shedding their developmental plasticity legacy (Rothenberg *et al.,* 2008).

T-cell development resembles that of B-cells, with few key differences. One difference is that while a given B-cell firstly expresses IgM, then both IgM and IgD, and later IgG or IgA or IgE (all with the same light chain), T-cells express either αβ or γδ-TCR during their whole life span, and usually react only with peptides from foreign antigens bound in the grooves of major histocompatibility complex proteins (MHC) (Yin *et al.,* 2011). During fetal development, T-cells express γδ-TCR. In the thymus, in the double-negative stage, developing thymocytes express both a γδ T-cell receptor and the pre-T-cell receptor composed of the TCRβ chain and the pre-Tα chain (pTα). In this stage, if the thymocyte receives signals through the γδ receptor, the cell is committed to the γδ lineage. This cell then matures into a γδ T-cell and migrates out of the thymus into peripheral circulation. On the other hand, if the developing thymocyte receives a signal through the pre-TCR, then the cell switches off the expression of the γδ-TCR, deletes the δ chain genes as a preliminary step to rearranging the TCRα- chain locus, and goes on to express a mature αβ T-cell receptor (Janeway *et al.,* 2001) (**Figure 31**).

T-cells recognize a "non-self" target only after antigens (small fragments of the pathogen) have been processed and presented in combination with a "self"-receptor, the MHC molecule. Briefly, these cells play an important role in both humoral and cellular immunity (Khajavi *et al.* 2003) by directly secreting soluble mediators or through cell contact-dependent mechanisms.

In the thymus, the progeny of T-cells is produced that generally display one of a vast repertoire of T-cell receptors (TCRs) (Kitchen *et al.,* 2012). Besides, during intrathymic maturation, T-cells also achieve specific T-cell markers, including CD3, CD4 or CD8

(Abbas and Lichtman, 2005), where double-positive αβ T-cells move into the cortico-medullary junction, undergoing positive and negative selection, and they mature into Th- and Tc-cells (Snyder, 2012).

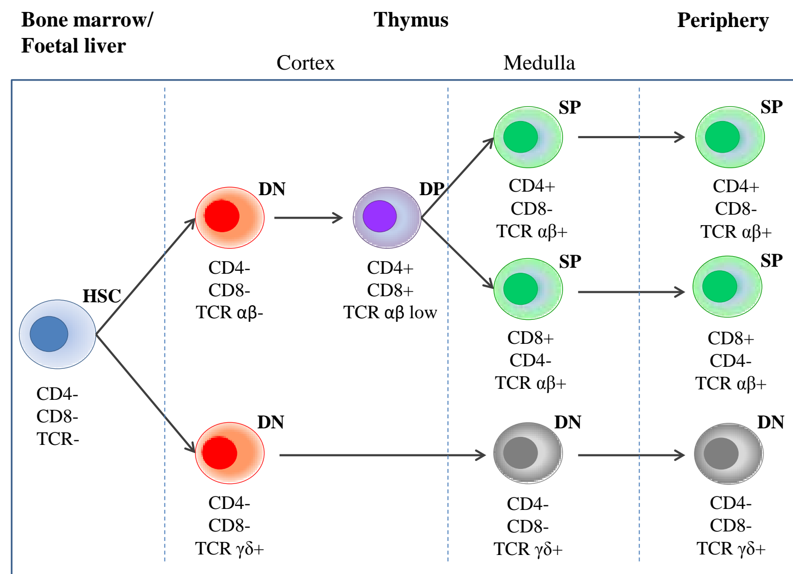


Figure 31. Maturation of αβ T-cells and γδ T-cells in the thymus. *DN*: Doble negative; *DP*: Doble positive; *HSC*: Hematopoietic stem cell; *SP*: Single positive; *TCR*: T-cell receptor.

Extrathymic T-cell commitment has also been detected in the spleens of mice under physiological conditions (Yang *et al.,* 2010). Moreover, recent studies have challenged the current dogma that thymocytes cannot self-renew, showing that thymocyte survival is determined by competition between incoming progenitors and resident cells (Peaudecerf *et al.,* 2012).

In secondary lymphoid tissues, they are located primarily in the paracortical regions of lymph nodes and the periarteriolar sheaths (PALS) of the spleen. These specific anatomic sites produce chemoattractant cytokines, for which T lymphocytes express receptors (Snyder, 2012).

There are several types of T-cells based on their specific function: helper, cytotoxic, memory, regulatory and gamma delta (γδ) T-cells (**Figure 32**).

- **Helper T-cells (Th)** secrete proteins called cytokines in response to antigenic stimulation to stimulate the proliferation and differentiation of T-cells, and also other cells, including B-cells, macrophages and other leukocytes (Abbas and Lichtman, 2005), which prove absolutely vital to the immune response.

- **Cytotoxic T-cells (Tc)** are responsible for cell-mediated immunity (Snyder, 2012). These cells enter peripheral tissues and directly kill infected and transformed cells, thereby protect the host from viral infections and cancer. Direct killing is mediated by secretion of perforin and granzymes, which cause apoptosis of target cells (Goldsby *et al.*, 2003).

- **Regulatory T-cells** inhibit immune responses by secreting immunosuppressive cytokines such as IL-10 and transforming growth factor-β (TGF- β) (Abbas and Lichtman, 2005).

- **Memory T-cells** are programmed to recognize and to respond to a pathogen once it has invaded. They reside in secondary lymphoid organs, mount recall responses to antigens, rapidly proliferate and differentiate into effector T-cells following antigen stimulation, and in peripheral tissues (Abbas and Lichtman, 2005).

- **γδ T-cells** are a minor subpopulation that recognizes that intact antigens are not bound to MHC receptors (Snyder, 2012).

- **Natural killer T-cells (NKTs)** perform both pro- and anti-inflammatory functions. Have been shown to modulate immune responses in several different settings, including cancer, autoimmunity, allergy, infection and graft versus-host disease (Abbas and Lichtman, 2005).

Although effector T-cells were originally considered to be terminally differentiated, it has been suggested that the phenotype of effector T-cells is not completely fixed, but is more flexible or plastic. T-cells can have 'mixed' phenotypes (that is, have characteristics that are usually associated with more than one T-cell subset) and can interconvert from one subset phenotype to another; however, instructive signaling can lead to the long-term fixation of cytokine memory. T-cell plasticity can be important for the adaptation of immune responses in different microenvironments and might be particularly relevant for host defense against pathogens that colonize different tissues.

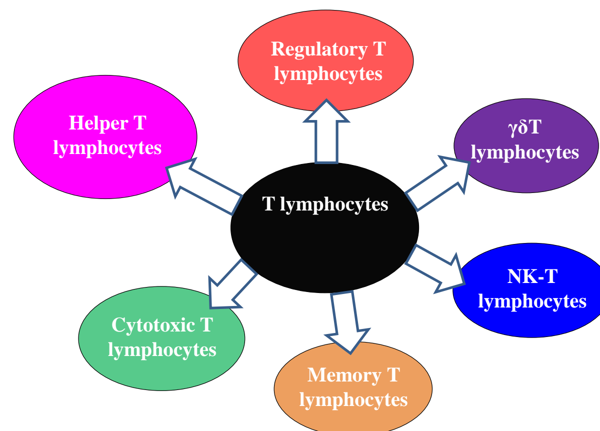


Figure 32. Subpopulations of T-lymphocytes: Helper T-cells, Cytotoxic T-cells, Regulatory T-cells, Memory T-cells, NK-T cells and γδ T-cells.

Flow cytometry is an adequate tool for determining the nature of these diverse cell populations. Each T-cell subset has a characteristic cytokine signature that can be measured at the single-cell level, combining surface phenotyping, surface markers or intracellular staining of cytokines and other signaling and effector molecules to analyze multiple parameters at once. However, it is difficult to find availability of antibodies of each lymphocyte subset in the rabbit species.

Helper T-cells (Th)

Helper T-cells, or "helpers", correspond to a subset of T lymphocytes whose classic marker of the membrane is the CD4 molecule. CD4 helper T-cells provide a peculiar example of apparently defined cell subsets at times described as lineages, but are also highly sensitive to tissue environmental cues that may change their fate (Mucida and Cheroutre, 2010). The discovery of and research into two other $CD4^+$ T-cell subsets, induced regulatory T- (iTreg) cells and Th17 cells, has led to rethinking the notion that helper T-cell subsets represent irreversibly differentiated endpoints (Zhou *et al.,* 2009; Mucida and Cheroutre, 2010; Zygmunt and Veldhoen, 2011). In fact depending on the received magnitude and patterns of TCR, co-stimulatory and cytokine signals, T-helper cells differentiate from Th0 into Th1, Th2, Th9 (Lu *et al.,* 2012), Th17, or Tfh cells (Kemeny, 2012), or other functional subsets including memory T-cells (**Figure 33**). However, major Th-cell subsets include Th1, Th2, and Th17.

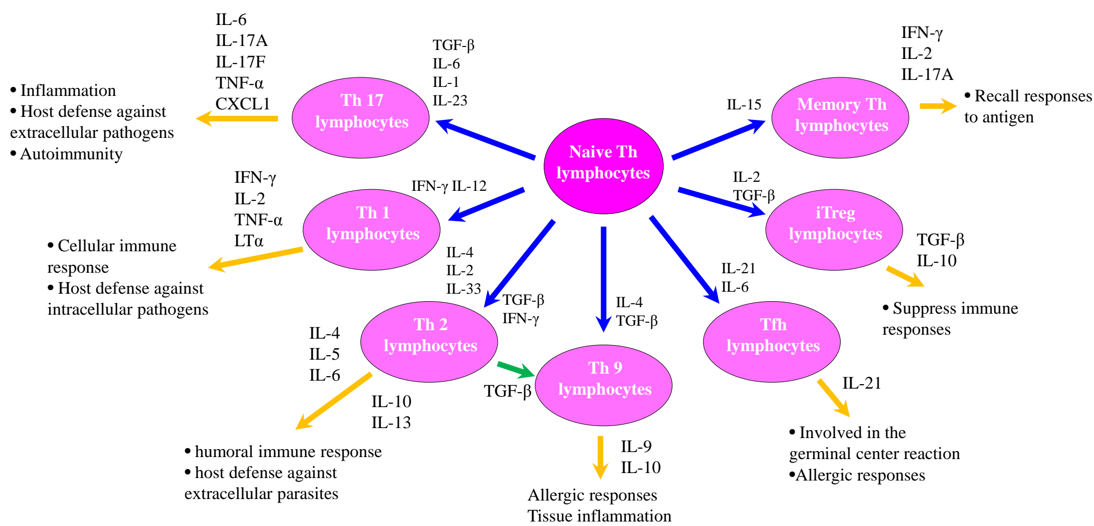


Figure 33. Different subsets of T helper lymphocytes. *CXCL*: Chemokine (C-X-C motif) ligand; *IFN*: Interferon; *IL*: Interleukin; *iTreg*: Induced or adaptive T regulatory; *LT*: Lymphotoxin. *Tfh*: T follicular helper; *TGF*: Tumor growth factor; *TNF*: Tumor necrosis factor.

They owe their name to the important role they play by collaborating with the responses of other immune system cells, such as cytotoxic T lymphocytes, macrophages, B lymphocytes and neutrophils, among others (Murphy *et al.*, 2008) (**Figure 34**). Therefore, they serve as managers by orchestrating a pleiotropy of immune activities against a wide variety of pathogens (Zygmunt and Veldhoen, 2011). Th-cells only recognize antigens coupled to Class II MHC molecules (Snyder, 2012) with the help of their expression of the CD4 co-receptor ($CD4^+$). Helper T lymphocyte lineage can be differentiated as:

Th0 lymphocytes

They are considered the precursor cells of Th1 and Th2 lymphocytes. The cytokines present in the medium allow subpopulations Th0 to be polarized to Th1 or Th2. In this way, when the Th0 lymphocyte is cultured in a medium with cytokines IL-12 and IFN-γ, they become Th1 lymphocytes. Moreover, when IL-4 is in the medium, the conversion of Th0 into Th2 lymphocytes is favored (Gomez-Lucia *et al.,* 2007).

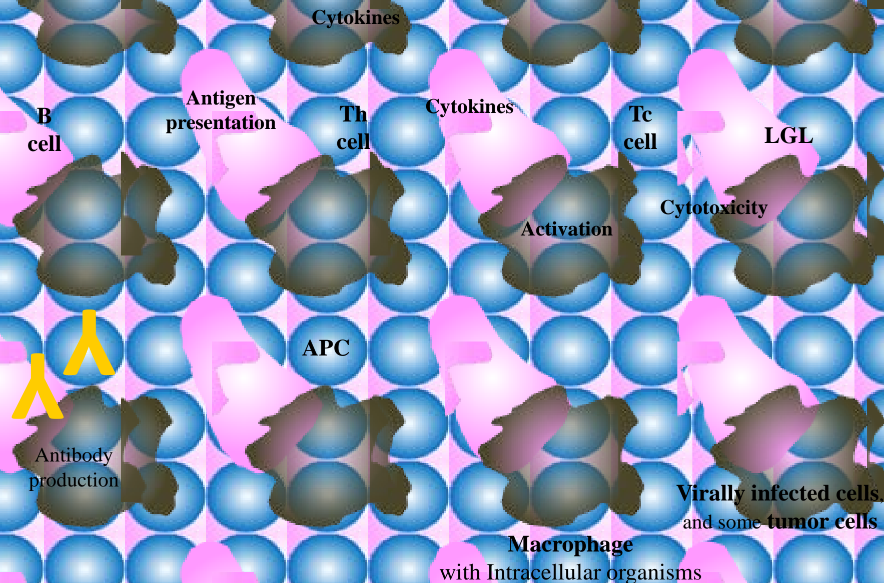


Figure 34.Functions of lymphocytes. T helper (Th) cells are stimulated by antigen presenting cells (APCs) and B-cells to produce cytokines, which control immune responses. Macrophages are activated to kill intracellular microorganisms. *LGL*: Large granular lymphocyte; *Tc*: T cytotoxic cells (Roitt *et al.,* 1996).

Th1 lymphocytes

Th1 lymphocytes are cells that are activated by cytokines IL-12 and IFN-γ, secreted by dendritic cells. IL-12 promotes a greater expansion and differentiation of Th1 lymphocytes. Activated Th1 lymphocytes produce IL-2, IFN-γ, TNF-α and lymphotoxin (TNF-β or LT-α). Secreted IL-2 by Th1 lymphocytes stimulates T lymphocytes proliferation, and also allows the activation of T and B lymphocytes and NK cells. IFN-γ stimulates Th1 lymphocytes and B-cell growth and IgG production, inhibits Th2 lymphocytes and activates macrophages. The main function of Th1 lymphocytes is the activation of macrophages. Thus, Th1 lymphocytes play a key role in the elimination of intracellular microorganisms. Macrophage activation by Th1 lymphocytes stimulates the potentiation of microbicidal mechanisms such as the formation of nitric oxide and superoxide to destroy bacteria. Furthermore, class II MHC molecules increase and, in turn, the macrophage antigen presentation to other T-cells also increases (Murphy *et al*., 2008).

Th2 lymphocytes

Th2 cells are cells that are potently induced mainly by the presence of IL-4 from undifferentiated CD4 T-cells. They produce IL-4, IL-5, IL-6, IL-10 and IL-13. The main function of Th2 cells is the activation and clonal expansion of B-cells and immunoglobulin production, especially of isotypes IgM, IgA and IgE. IL-4, IL-5, and IL-13 are growth factors for B-cells, while IL-10 inhibits macrophage activation (Gomez-Lucia *et al.,* 2007; Murphy *et al*., 2008).

Besides, Th2 lymphocytes are involved in the change of the class of immunoglobulin to IgE, which is particularly important in allergic processes and immunity against parasites (Gomez-Lucia *et al.,* 2007).

Th17 lymphocytes

A subpopulation of CD4 lymphocytes, characterized by the ability to produce IL-17, is designated as Th17 lymphocytes (Snyder, 2012).

Th17 lymphocytes were first described as orchestrators of neutrophil recruitment and activation, and as key players in chronic inflammation and autoimmunity. More recent evidence suggests that Th17 lymphocytes and their effector cytokines play a crucial role in maintaining mucosal immunity and barrier integrity, including skin, lung and gut (Rendon and Choudhry, 2012).

Differentiation of these cells is driven by IL-6, IL-1, TGF-β and IL-23 (Snyder, 2012). These cells are characterized by their secretion of IL-6, IL-17A, IL-17F, TNF and CXCL1 (Onishi and Gaffen, 2010).

Cytotoxic T-cells (Tc)

Cytotoxic T lymphocytes (killer) represent a lymphocyte population that has membrane marker $CD8^+$ and, like Th lymphocytes, the TCR-CD3 complex and other functionally important molecules such as CD2 and LFA-1. Its main effector response is directed to eliminate virus-infected cells or cancer cells (Gomez-Lucia *et al.,* 2007). T-cell activation is tightly controlled and generally requires a very strong MHC/antigen activation signal, or additional activation signals provided by helper T-cells.

Tc-cells recognize the peptides bound to MHC molecules of class I to which TCR is recognized by its receptor. After activation, they are expanded due to the IL-2 secreted by Th1 cells, thereby increasing the number of effector cells against a particular antigen (Gomez-Lucia *et al.,* 2007). To exert their cytotoxic function, they release lymphotoxins, which cause cell lysis through the following mechanisms:

- Perforins: a molecules C9-like component of the complement system, whose function is to generate pores in the membrane of target cells, thus allowing ions, water and toxins to enter.

- Granzymes: enter the target cell through the pores generated by perforin. They activate procaspase 3 and 8 in target cells by activating their apoptosis. Currently, granzymes A, B, H, K and M have been recognized.

- Granulysins: molecules similar to perforins that pierce the membrane of target cells, inducing them to undergo apoptosis.

- Fas Ligand (FasL, CD95L, CD178): its ligand, the Fas molecule (CD95), is expressed in cells under stress (for example, virus-infected cells or cancer cells). The interaction between Fas and its ligand induces apoptosis in target cells (Gomez-Lucia *et al.,* 2007).

Regulatory T-cells

Different types of regulatory T-cells, which express distinct cytokines or receptors and function by diverse pathways at differing stages of the immune response, have been described: Regulatory T-cells type 1 (Tr1); T helper 3 (Th3); $CD8^+$ regulatory T-cells;

Double negative regulatory T-cells; γδ regulatory T-cells; Natural killer regulatory T-cells and $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T (Treg) cells (**Figure 35**).

Treg, also known as suppressor T-cells, are a subpopulation of $CD4^{+}$ T lymphocytes that have been found to maintain self-tolerance and control excessive immune responses to foreign antigens (Shalev *et al*., 2011).

Foxp3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors, in Treg cells has been described as a master switch or regulator for Treg cell development and function (Shalev *et al*., 2011).

As a result of the suppressive effects on $CD4^{+}$ and $CD8^{+}$ effector T-cells, Treg controls the adaptive immune system and prevents autoimmunity. In addition, it inhibits B lymphocytes, dendritic cells and monocytes/macrophages. It is interesting to note that several recent research works have shown that $CD4^{+}CD25^{+}Foxp3^{+}$ is also able to inhibit NK cells. Thus, Treg exerts its control on immune responses from the onset (triggering innate immune cells) to the effector phase of adaptive immunity (B- and T-cell-mediated responses) (Ralainirina *et al.,* 2007).

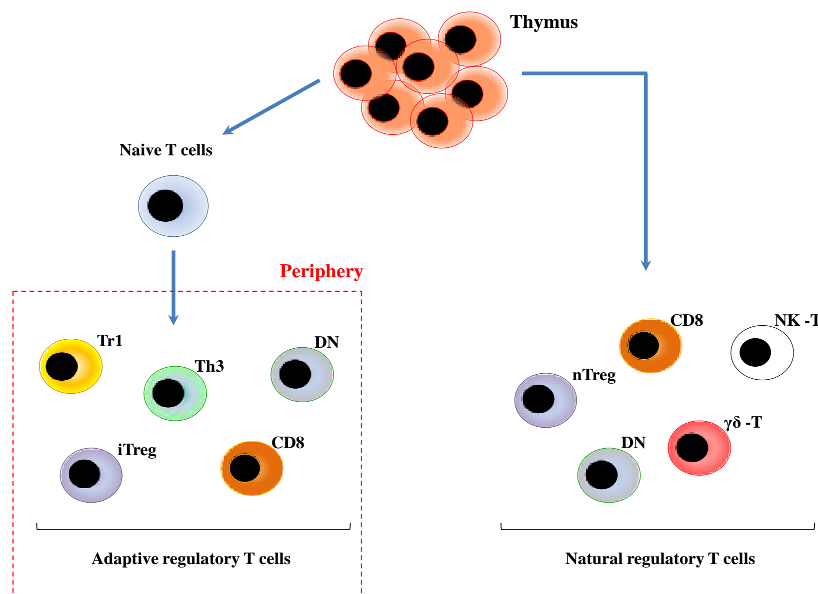


Figure 35. Development of regulatory T-cells. *iTreg*: Induced regulatory T-cell; *DN*: Doble negative; *NK-T*: Natural killer T-cell; *nTreg*: Natural regulatory T-cell.

Regulatory T-cells comprise two major subsets based on presumed ontogeny: adaptive regulatory T-cells, which are induced in the periphery in response to antigen stimulation under tolerogenic conditions, and naturally-occurring regulatory T-cells, which are constantly produced by the thymus (Shalev *et al*., 2011). In this way, Treg may be grouped into two subgroups:

- *Natural regulatory T-cells (nTreg)* are $CD4^{+}$ cells expressing higher levels of the α chain of the receptor for IL-2 (CD25) and are, therefore, $CD4^{+}CD25^{+}$ and the only ones expressing CD25 when they are virgin. nTreg lymphocytes are activated through transcriptional repressor FoxP3, which controls their development (Snyder, 2012).

Transcriptor FoxP3 has not only transactivator effects (positive transcriptional regulation) to induce the increased expression of CD25, CTLA-4 (antigen 4 cytotoxic T lymphocyte-associated) and the GITR receptor (a tumor necrosis factor induced by glucocorticoids), but also transrepressor effects (regulation negative transcriptional) that inhibit the production of IL-2, NFAT (the nuclear factor of activated T-cells) and NF-κβ (nuclear factor Kappa Beta). Transcriptor FoxP3 interferes with the interaction between AP-1 (activating protein 1) and NFAT in the promoter of IL-2, which prevents the transcriptional activation of the IL-2 gene and, consequently, leads to reduced IL-2 production (Gasteiger and Kastenmuller, 2012). In the absence of regulation, $CD4^{+}CD25-$ cells produce high levels of IL-2, which is involved in increasing the autoimmune response, and also in the activation of $TCD8^{+}$ and NK cells (Gonzalez *et al.,* 2010).

nTreg cells are potentially autoreactive, express the αβ-TCR receptor and are apparently selected in the thymus by high affinity binding to MHC class II with own peptides. In this way, these cells evade thymic clonal deletion which, when activated by autoantigens, do not differentiate into cells capable of generating an autoimmune response, but differ in potent suppressor cells capable of inhibiting other autoreactive cells. This process is known as regulatory tolerance or dominant immune suppression (Murphy *et al*., 2008).

- *Adaptive regulatory T-cells or inducible (iTreg)* develop outside the thymus in the periphery from $CD4^{+}$ undifferentiated cells and are essential to maintain a non inflammatory environment in gut, to suppress immune responses to environmental and food allergens, and to decrease chronic inflammation (Curotto de Lafaille and Lafaille, 2009). This group can be divided into two subpopulations that do not express higher levels of the α chain of the receptor for IL-2 (CD25) or Foxp3:

  - Th3 cells: produce transforming growth factor beta (TGF-β), which blocks cytokine production by T-cells, cell division and cytolytic capacity (Murphy *et al.,* 2008).

  - Tr1 cells: unlike Th3 cells, cytokine production is limited to TGF-β, which allows them to differentiate from the above-mentioned cells, Tr1 cells also produce IL-10, and IL-5. IL-10 suppresses the T-cell responses directly by reducing the production of IL-2, TNF-α, and IL-5 by T-cells, and by indirectly inhibiting antigen presentation by reducing the expression of MHC molecules and molecules co-stimulatory by antigen-presenting cells (Murphy *et al.,* 2008).

  TGF-β is important for the establishment of immunological tolerance, at least in part, because it is required for the differentiation of iTreg cells and for the maintenance of nTreg cells after they emigrate from the thymus (Marie *et al.,* 2005; Li *et al.,* 2006). However in the case of adaptive regulatory T-cells (Tr1 cells), their development is also favored by the relative abundance of IL-10 (Murphy *et al.,* 2008).

Treg lymphocytes are capable of preventing the activation and proliferation of CD4 T helper cells, CD8 T cytotoxic cells and B lymphocytes and are, therefore, a key element in immune system regulation (Taams *et al.,* 2005; Murphy *et al.,* 2008). The mechanisms by which they allow the control of immune activity are summarized in the following points:

- ✓ The direct suppression mechanism: it is conducted by cell-cell contact and is associated with increased CTLA-4 molecules. Treg lymphocytes exhibit a high expression of CTLA-4, which provides inhibitory signals resulting in the inactivation of effector cells (Murphy *et al.,* 2008).

- ✓ The indirect suppression mechanism: it takes place by the secretion of immunosuppressive cytokines (Taams *et al.,* 2005).

Moreover, Treg cells can secrete perforin and granzyme, and they induce the apoptosis of effector lymphocytes. In addition, Treg lymphocytes secrete galectin-1, which is able to induce apoptosis in T lymphocytes (Taams *et al.,* 2005; Murphy *et al.,* 2008).

γδ T-Cells

In mammals, T-cells develop along two discrete pathways characterized by the expression of either αβ or γδ T-cell receptors (Massari *et al.,* 2012). In most species, a minority of T lymphocytes expresses γδ-TCR. However in young ruminants, sheeps and pigs, they constitute a high proportion, reaching 60% of total T lymphocytes. These lymphocytes present γδ-TCR (TCR1) that differs from other lymphocytes presenting αβ-TCR (TCR2) and are negative for both CD4 and CD8 (Tizard, 2009). In all these species, the genomic organization of the T-cell receptor gamma (TRG) locus is well-known. Recent studies have defined the genomic organization of the TRG locus in rabbits, another mammalian γδ high species, as deduced from the genome assembly (Massari *et al.,* 2012).

γδ T-cells develop in the thymus and migrate to the epithelium of skin and intestine, mammary glands and reproductive organs. Although these cells can be found within regional lymph nodes and the lamina propria, they primarily reside in these organs as intraepithelial lymphocytes (IELs) (Snyder, 2012). Although a precise function remains unknown, they seem to be a component of adaptive immunity as they rearrange TCR genes to produce receptor diversity, and they can also develop a memory phenotype; moreover, the various subsets also form part of the innate immune system as restricted TCR or NK receptors may be used as pattern recognition receptors (Tizard, 2009).

Two subpopulations have been reported (Rogers *et al.,* 2005):

- $WC1^+$ γδ T-cells, which are involved in innate immunity

- $WC1^-$ γδ T-cells, which perform various regulatory functions.

2.4. Immune response

The purpose of the immune response is both the non specific and specific defense of an individual's biological integrity by acting as a system to maintain the homeostasis of the organism. Any immune response firstly involves the recognition of the pathogen or other foreign material, and secondly the development of a reaction against it to eliminate it. Different immune response types fall into two main categories: innate (or non adaptive) immune responses; adaptive immune responses (Roitt *et al.,* 1996). Akira (2012) represents the two main categories of immune response and their interactions simplely in **Figure 36**.

The main differences between these two responses are summarized in the **Table 3**.

Table 3. A summary of properties distinguishing the innate and adaptive immune system (Gonzalez *et al.,* 2011).

Property	Defense mechanism (innate)	Immune system (adaptive)
Present in:	All organisms	Vertebrates only
Self-non-self discrimination is:	Innate	Somatically selected
Receptors are:	Germline selected	Somatically selected
Antiself selection purges:	Germline selected	Cells
Defects in antiself selection:	Individuals	Causes autoimmunity
Unresponsive to the:	Does not causes autoimmunity	Self-of-the-individual
Effector mechanisms are:	Self-of-the-species	Coupled with innate effector mechanisms

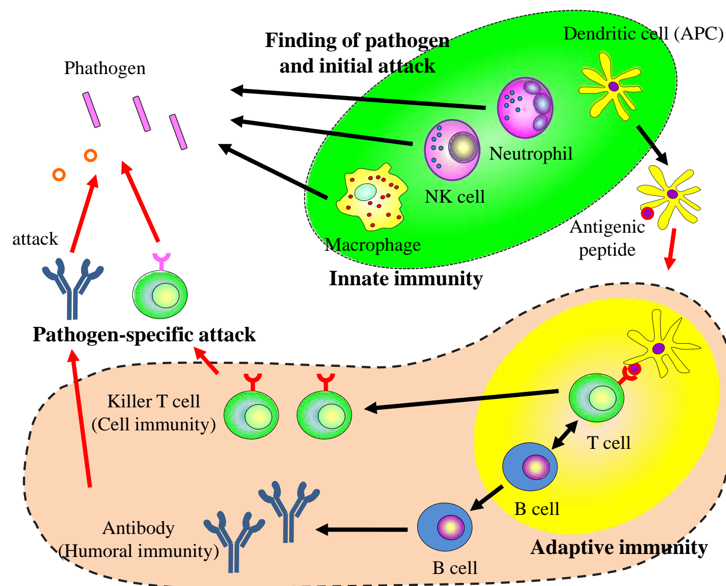


Figure 36. Interactions of innate and adaptive immunity. *APC*: Antigen presenting cell (Akira, 2012).

2.4.1. Innate immune response

The innate immune system is an ancient, broad-spectrum defense system found in all eukaryotes (McCann *et al.,* 2012), and is the dominant immune system in plants, fungi, insects and primitive multicellular organisms (Murphy *et al.,* 2008).

In vertebrates, this system consists of inflammatory cells and soluble mediators that comprise the first line of defense against microbial infection and, importantly, they trigger antigen-specific T- and B-cell responses leading to lasting immunity (Oberbarnscheidt *et al.,* 2011).

The innate immune system recognizes and responds to pathogens generically. However, it is not absolutely correct to denote it as non specific, as is often the case. Although certain aspects of innate immunity are non specific, it is increasingly appreciated that multicellular organisms have evolved specific recognition mechanisms, designated pattern recognition receptors (PRRs), as part of their innate immune defense to trigger clearance of pathogens (Monack, 2011). Unlike the adaptive immune system, it does not confer long-lasting immunity to the host. Nonetheless, it provides immediate defense against infection and has

been considered to represent a temporary system until adaptive immune responses can be triggered (Akira, 2012).

Conversely to the antigen receptors of the adaptive immune system, which are totally “unfocused”, the receptors of the innate system are precisely tuned to detect the presence of common pathogens (disease-causing agents) presented in daily life viruses, bacteria, fungi and parasites, and to also have receptors that detect when “uncommon” pathogens kill host cells. Consequently, the innate system is responsible for sensing danger and for activating the adaptive immune system (Sompayrac, 2012).

These specific microbial components, known as pathogen-associated molecular patterns (PAMPs), are essential for the pathogen’s life cycle. However, these PAMPs are either absent or compartmentalized inside the host cell. Moreover, they are sensed by the host´s germline encoded PRRs (Kumar *et al.,* 2011), which are expressed in innate immune cells, such as dendritic cells, macrophages and neutrophils (Medzhitov, 2007; Blasius and Beutler, 2010; Kawai and Akira, 2010; Takeuchi and Akira, 2010), and they sense various molecule classes, including proteins, lipids carbohydrates and nucleic acids (Medzhitov, 2007; Kumar *et al.,* 2009a; Blasius and Beutler, 2010; Kawai and Akira, 2010; Takeuchi and Akira, 2010). Both the innate immune and adaptive immune systems rely heavily on the function of PRRs. However, recognition of non-pathogenic and commensal microflora by PRR remains largely unknown (Ichinohe *et al.,* 2011). Presumably, compartmentalization (confinement of commensal microflora to the luminal side of the intestinal epithelium), transforming growth factor (TGF-β) and interleukin (IL-10) play an important role in this process. PAMPs are highly conserved throughout evolution and are less destined for mutation since they are vital for the survival of microbes.

Three main features of PAMPs make them ideal targets for the innate immune system: first, they are produced only by microbes and not by host cells; second, they are invariant between microorganisms of a given class; and third, PAMPs are essential for the survival of the microbial flora (Wells *et al.,* 2011).

Different PRRs generally recognize diverse ligand specificities. The broad specificities of PRRs and their ability to form functional multireceptor complexes allow large combinatorial repertoires. This further diversifies the recognition and signaling of cooperating PRRs, and enables the host to detect almost any pathogen type, to discriminate between different microorganisms and to develop a competent immune response (Aoki *et al.,* 2012). There are a variety of PRRs types classified as secreted, transmembrane and cytosolic forms (Snyder, 2012), which fall into three broad functional groups as follows:

- Cell-based PRRs, which are active pro-inflammatory signaling pathways. These result in the induction of genes to produce anti-microbial peptides, lysozymes, inflammatory cytokines and chemokines. They also increase the expression of the molecules involved in the activation of adaptive immunity.

- Cell-based PRRs, which stimulate phagocytic responses in macrophages, neutrophils and dendritic cells. They bind microbial PAMPs and mediate the phagocytosis or endocytosis process. In macrophages and dendritic cells, internalized microbes are then processed into peptides for presentation to T-cells by MHC molecules.

- Fluid-based PRRs, which form part of the humoral arm of the innate immune system. They are recognized by the receptors expressed in cells of the innate immune system. Members include collectins, ficolins and pentraxins.

Toll-like receptors (TLRs) and C-type lectins are examples of transmembrane PRRs, and they have a limited distribution, including macrophages, NK cells and dendritic cells (Snyder, 2012).

TLRs are the most widely studied PRRs and are considered to be the primary sensors of pathogens (Kumar *et al.,* 2011). Toll-like receptors are expressed in different cell types, including monocytes, macrophages, eosinophils, mast cells and dendritic cells. Some can recognize extracellular proteins, lipoproteins and lipopolysaccharides, while others recognize intracellular viral nucleic acids (Gomez-Lucia *et al.,* 2007; Tizard, 2009). Initially, they were identified in *Drosophila*, but currently there are 13 known homologs of Toll, 10 of which are expressed in mammals (**Table 4**).

Table 4. A summary of pathogen-associated molecular patterns (PAMPs) recognized by Toll-like receptors (TLRs) (Tizard, 2009).

TLR receptor	PAMPs recognized
TLR1	diacetylated lipoproteins
TLR2	Peptidoglycan, lipoprotein, zymosan, spirochetes, heat shock proteins
TLR3	Viral RNA
TLR4	LPS, viral proteins, lipoteichoic acid, heparan sulphate
TLR5	Flagellin
TLR6	Necrotic cells, diacetylated lipoproteins
TLR7	Viral RNA
TLR8	Viral RNA
TLR9	Bacterial DNA
TLR10	A pseudogene

TLRs can be found as either individual transmembrane units or in pairs with a range of accessory signaling molecules. Most TLR signaling occurs through a TIR domain-containing adaptor, such as Myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF) and Toll-like receptor adaptor molecule (TRAM). Downstream signaling regulates inflammatory cytokine production and the co-stimulatory receptor expression via the NF-kappa B pathway (**Figure 37**). When Toll-like receptor 4 (TLR4) was discovered, it was observed that a dominant active form of TLR4 was able to stimulate NF_kB and NF_kB-dependent inflammatory genes, and that TLR4 induced the expression of CD80 (Medzhitov *et al.,* 1997; Uematsu and Akira, 2008).

Expression of the CD80 co-stimulatory molecule is necessary for the activation of naive T lymphocytes. Its induction by TLR4 was the first evidence that the innate immune system could participate in the polarization of naive T lymphocytes and could activate adaptive immunity (Medzhitov *et al.,* 1997).

TLR 4 is recognized as the main lipopolysaccharide (LPS) receptor (Kvarnhammar and Cardell, 2012). Kumar *et al*. (2005) represent the binding of LPS to TLR4 simply in **Figure 38**. By promoting the release of inflammatory cytokines, LPS also mobilize the innate and adaptive immune response (Beutler, 2000).

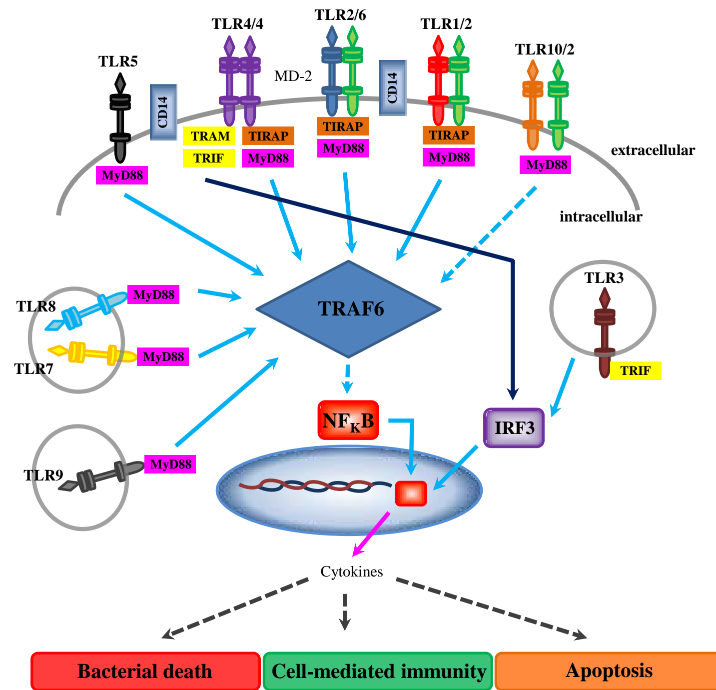


Figure 37. Toll-like receptors signalling. *IRF3*: Interferon regulatory factor 3; *MD2*: Lymphocyte antigen 96; *MyD88*: Myeloid differentiation primary response gene (88); *TIRAP*: Toll-interleukin 1 receptor (TIR) domain containing adaptor protein; *TLR*: Toll-like receptors; *TRAF*: Tumor necrosis factor receptor associated factor; *TRAM*: Toll-like receptor adaptor molecule; *TRIF*: TIR-domain-containing adapter-inducing interferon-β; *NF$_{k}$B*: Nuclear factor-kappa B.

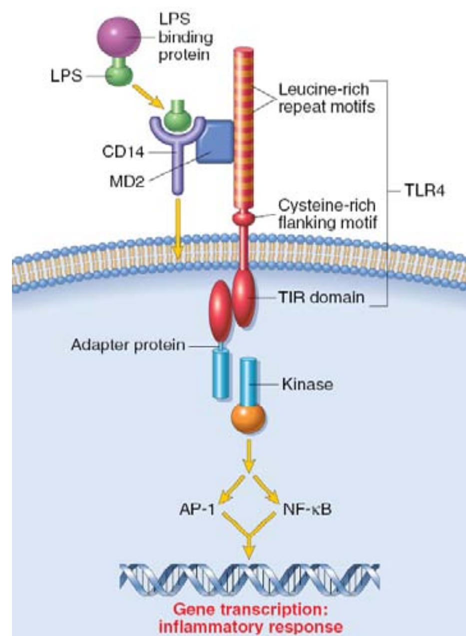


Figure 38. The binding of lipopolysaccharide (LPS) to TLR4 results in the activation of a signal transduction pathway, leading to gene transcription and the elicitation of an inflammatory response. *AP-1:* Activating protein 1; *MD2*: Lymphocyte antigen 96; *NF-κB*: Nuclear factor-kappa B; *TIR:* Toll/interleukin-1 receptor; *TLR*: Toll-like receptor (Kumar *et al.,* 2005).

Activation of TLRs by PAMPs leads to the induction of an inflammatory cascade, and to the activation of both the innate and adaptive arms of the immune response (Chang *et al.,* 2012). In the macrophages context, TLRs not only regulate cytokine production, but also enhance the expression of the antigen presentation and co-stimulatory molecules that facilitate pathogen clearance and the induction of an adaptive immune response (Gabhann and Jefferies, 2011). In fact, PAMP recognition also facilitates the optimal development of adaptive immune responses by APC, while ensuring that enhanced antigen-specific responses occur only when a pathogen is present (Strutt *et al.,* 2010).

Among the major components of innate immunity, we find intact epithelial barriers, phagocytic cells, natural killer (NK) cells, and a number of plasma proteins, the most important of which are the proteins of the complement system (Snyder, 2012).

2.4.1.1. Components of innate immunity

Innate immune response barriers

A variety of host defense mechanisms have been developed by the organism which, in most cases, effectively prevent the development of invasive microbial disease (Albiger *et al.,* 2007), control resident colonizing microflora and eliminate pathogens. These defense mechanisms are the result of anatomic (e.g., skin, mucosal epithelia, cilia) and physiologic (e.g., stomach pH, body temperature) properties, and phagocytic and inflammatory responses (Snyder, 2012).

Among these defense mechanisms, skin and mucous membranes provide physical and chemical barriers to infection by entrapping the foreign pathogens and cilia propelling them out of the body (Mayer, 2006). Skin protects the host against infection through a variety of mechanisms. Antimicrobial peptides (AMPs) are major contributors to cutaneous innate immunity, and this system, combined with the unique ionic, lipid and physical barrier of the epidermis, is a first-line defense against invading pathogens (Gallo and Nakatsuji, 2011), while the mucosal system is the first line of defense against many pathogens. A homeostatic environment between commensal microbiota and pathogenic infections is established in the host (Mangan and Fung, 2012). By the cough reflex, respiratory mucosa, with its ciliary apparatus, can move in a retrograde fashion to enter the respiratory tree. Respiratory mucosa also possesses a mucous secretion, whose main component is mucin, which protects it from pathogens (Murphy *et al.,* 2008).

Physiological barriers include body temperature, pH and other body secretions that prevent any microorganisms from growing and entering the body. Moreover, the most noteworthy antibacterial substance, lysozyme, is present in tears (Mayer, 2006), which fragments the muramic acid present in bacteria membranes (Kozar *et al*, 2000). Following infections and inflammatory events, a rapid innate immune response is evoked to dampen inflammatory processes.

Phagocytic cells

Another important innate defense mechanism is ingestion of extracellular particulate material by phagocytosis. Phagocytosis is a type of endocytosis, the general term for uptake by a cell of the material from its environment. In phagocytosis, a cell's plasma membrane expands around the particulate material, which may include whole pathogenic

microorganisms, to form large vesicles called phagosomes. Most phagocytosis is conducted by specialized cells, such as blood monocytes, neutrophils and tissue macrophages (Kindt *et al.,* 2007).

Natural killer (NK) cells

NK cells are considered prototypical innate immune cells. However, recent discoveries have tended to refine the dogmatic concepts of innate and adaptive immunity. In many ways, NK cells are highly related to T-cells and represent the closest innate immune cell lineage to adaptive immune cell populations (Narni-Mancinelli *et al.,* 2011). Natural killer (NK) cells are important players in innate immunity, and are not only dedicated to the host defense against viruses, but are also involved in the immune surveillance of tumors, as previously described.

Complement system

The complement system is a major component of innate immunity, and consists of both soluble factors and cell surface receptors (Stoermer and Morrison, 2011). A variety of specific and non specific immunological mechanisms can convert inactive forms of complement proteins into an active state that is able to damage the membranes of pathogenic organisms by either destroying pathogens or facilitating their clearance (Kindt *et al.,* 2007).

Reactions between complement molecules, or fragments of complement molecules, and cellular receptors trigger the activation of cells of innate or adaptive immune systems (Kindt *et al.,* 2007). The activation and regulation of the complement system are described in a number of recent review articles (Gros *et al.,* 2008; Ziupfel and Skerka, 2009; Ricklin *et al.,* 2010). The system can be activated through three pathways: classical, alternative and lectin (Ballanti *et al.,* 2011). Nonetheless, recent novel complement activation pathways have been identified (Huber-Lang *et al.,* 2006; Kang *et al.,* 2006; Kemper *et al.,* 2010). The classical complement pathway has been described to be activated by IgM and certain IgG isotypes bind to the antigen; the alternative one is activated by the spontaneous hydrolysis of C3; finally, the lectin pathway is triggered by pattern recognition receptors, such as mannose-binding lectin and ficolins (Stoermer and Morrison, 2011). Janeway *et al.* (2001) represent the three pathways of complement activation simply in **Figure 39**.

The complement system acts as a rapid, efficient immune surveillance system that has distinct effects on healthy and altered host cells and on foreign intruders (Ricklin *et al.,* 2010). The final result of this activation cascade is the generation of active components, including C3b and C4b, which help the assembly of enzymes called C3/C5-convertases that, in turn, facilitate the downstream cleavage and formation of the membrane attack complex (MAC), and are capable of lysing pathogens (Bernet *et al.,* 2011).

Additionally, activation products C3a and C5a show anaphylatoxic and chemotactic properties (Volanakis, 1998), and also play a role in T-cell activation (Strainic *et al.,* 2008). Moreover, the surface-bound complement components deriving from C3 interact with specific immune receptors to act as a connecting link with the adaptive immune system (Carroll, 2004). Hence, the complement system exerts assault on pathogens directly by lysis and indirectly by boosting pathogen-specific immune responses (Pyaram *et al.,* 2010). In fact, recent studies on collectins indicate that these surfactant proteins may kill certain

bacteria directly by disrupting their lipid membranes or, alternatively, by aggregating bacteria to enhance their susceptibility to phagocytosis (Kindt *et al.,* 2007).

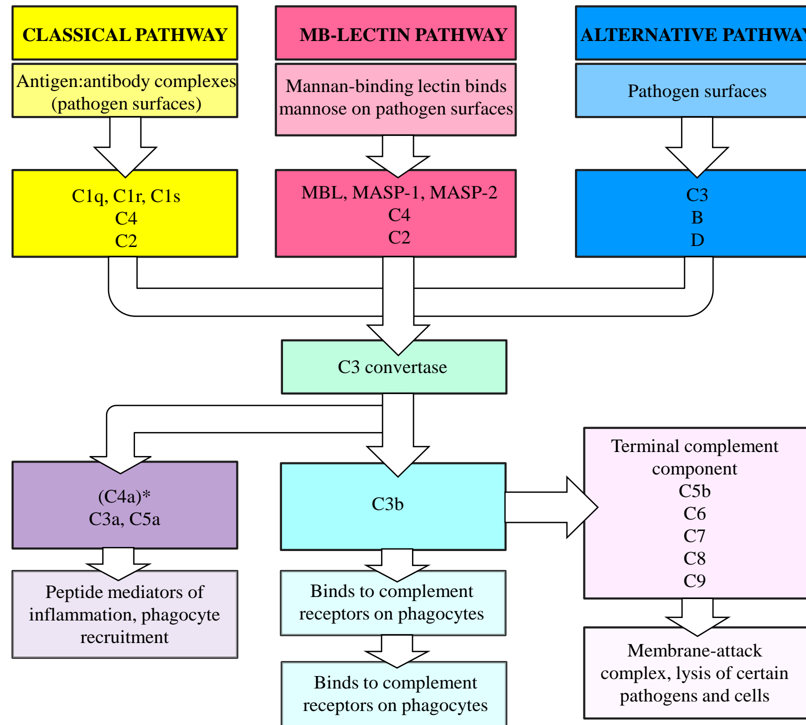


Figure 39. The early events of all three pathways of complement activation involve a series of cleavage reactions that culminate in the formation of an enzymatic activity called a C3 convertase, which cleaves complement component C3 into C3b and C3a. The production of the C3 convertase is the point at which the three pathways converge and the main effector functions of complement are generated. C3b binds covalently to the bacterial cell membrane and opsonizes the bacteria, enabling phagocytes to internalize them. C3a is a peptide mediator of local inflammation. C5a and C5b are generated by cleavage of C5b by a C5 convertase formed by C3b bound to the C3 convertase (not shown in this simplified diagram). C5a is also a powerful peptide mediator of inflammation. C5b triggers the late events in which the terminal components of complement assemble into a membrane-attack complex that can damage the membrane of certain pathogens. C4a is generated by the cleavage of C4 during the early events of the classical pathway, and not by the action of C3 convertase, hence the *; it is also a peptide mediator of inflammation but its effects are relatively weak. Similarly, C4b, the large cleavage fragment of C4 (not shown), is a weak opsonin. Although the classical complement activation pathway was first discovered as an antibody-triggered pathway, it is now known that C1q can activate this pathway by binding directly to pathogen surfaces, as well as paralleling the MB-lectin activation pathway by binding to antibody that is itself bound to the pathogen surface. *MASP:* Mannan-binding lectin-associated serine protease (Janeway *et al.,* 2001).

Besides its involvement in eliminating microbes, the complement system is recognized as participating in diverse processes such as synapse maturation, clearance of immune complexes, angiogenesis, and mobilization of hematopoietic stem cells, tissue regeneration, and lipid metabolism (Ricklin *et al.,* 2010).

Enzymes and others molecules

Enzymes participate actively in oxygen-dependent bactericidal activity. This process involves hydroxyl radicals, the myeloperoxidase enzyme, superoxide dismutase, and many

halogenated compounds. Other important enzymes are transferrin, lactoferrin, lysozyme and ceruloplasmin. In the cytoplasm of polymorphonuclear cells, there are granules containing proteases, hydrolytic enzymes, lysozyme and other series of enzymes capable of destroying many microorganisms (Abbas and Lichtman, 2005). Lactoferrin has the ability to avidly bind iron by depriving the bacteria of this micronutrient required for normal metabolism (Tizard, 2009).

Molecules, such as cytokines, are important in the immune response as they display the differentiation of lymphocyte populations, stimulation of immune cells, intercellular communication, etc., among other activation functions (Rojas, 2004). All cytokines are proteins or peptides, some with sugar molecules attached (glycoproteins) (Roitt *et al.,* 1996). The principal sets of cytokines are interferons (IFNs). Interferons are molecules that induce an antiviral defense status, differentiation, activation of T-cells and macrophages, and display anti-tumoral activity (Rojas, 2004). One group of interferons (IFNα and IFNβ) is produced by cells which have become virally infected, while another group is released by certain activated T-cells (IFNγ) (Roitt *et al.,* 1996). Another set of cytokines is interleukins (ILs) are produced mainly by T-cells, while others are produced by mononuclear phagocytes or tissue cells. Their functions are to stimulate other cells in order to divide and differentiate, such as colony-stimulating factors (CSFs), which are involved in directing the division and differentiation of BM stem cells and the precursors of blood leucocytes. Furthermore, tumor necrosis factors (TFNα and TFNβ) and transforming growth factor-β (TGFβ) are important in mediating inflammation and cytotoxic reactions (Roitt *et al.,* 1996).

Chemokines are substances that stimulate the migration of polymorphonuclear leukocytes into tissue where they must act and mediate the amnestic response (i.e., monocyte chemotactic factor (MCP), and chemotactic factor for eosinophils (ECF)) (Abbas and Lichtman, 2005).

2.4.2. Adaptive immune response

Innate immune recognition is based mainly on a series of germline-encoded receptors that have been selected by evolution to recognize the non-self molecules present in microorganisms. Innate immunity also recognizes changes in cells caused by infection, such as lack or induction of self-molecules (Gonzalez *et al.,* 2011). Non specific defense mechanisms provide a good protection system but, in many cases, do not suffice to effectively defend the body. Fortunately, there is a specific immune response.

The specific immune defense mechanism has evolved much more than innate immunity, and can recognize and react to a large number of infectious and non-infectious agents stimulated after exposure to these agents; thus, each successive exposure to a foreign agent increases their defensive intensity (Gomez-Lucia *et al.,* 2007).

The main features of the adaptive immune response are: specificity, diversity, memory, expertise, self-limitation and immune tolerance (Abbas and Lichtman, 2005).

The specific or adaptive immune response begins by preliminarily recognizing the foreign element, irrespectively of whether it is present in host cells (virus, bacteria or intracellular parasites) or in extracellular fluids by lymphocytes and other cells such as dendritic cells, macrophages, etc. (Gonzalez *et al.,* 2011). Adaptive immunity somatically generates large

repertories of receptors on T and B lymphocytes (T-Cell Receptor [TCR]) and B-Cell Receptor [BCR]), which may be able to virtually recognize any non-self antigen. The generation of these large repertoires of adaptive receptors for non-self antigens has created two new problems during evolution. First, this random process may yield some receptors with good avidity for self-molecules. Second, the adaptive immune system has to learn how to discriminate self from non-self in order to avoid an anti-self reaction (Gonzalez *et al.,* 2011). In order to facilitate this discrimination, the adaptive immune system brings into play the membrane receptors of cells and, in particular, the molecules of the major histocompatibility complex (MHC). Thus, the immune system discriminates self from non-self during adaptive immunity in the periphery by not recognizing the structural differences between self and foreign antigens, but by perceiving the avidity of T-cell activation (Wu *et al.,* 2009), besides also providing important feedback during acute inflammatory processes to provide host defense or to limit pathology (Kelly-Scumpia *et al.,* 2011).

Adaptive immunity generally consists in cell-mediated immunity mediated by T lymphocytes against intracellular pathogens, mainly against viruses, to also prevent the emergence and growth of tumor cells and humoral immunity mediated by B lymphocytes against extracellular pathogens and toxins (Synder, 2012). Drouet-Viard and Fortun-Lamothe (2002) represent the interactions between adaptive and humoral immunity in **Figure 40**.

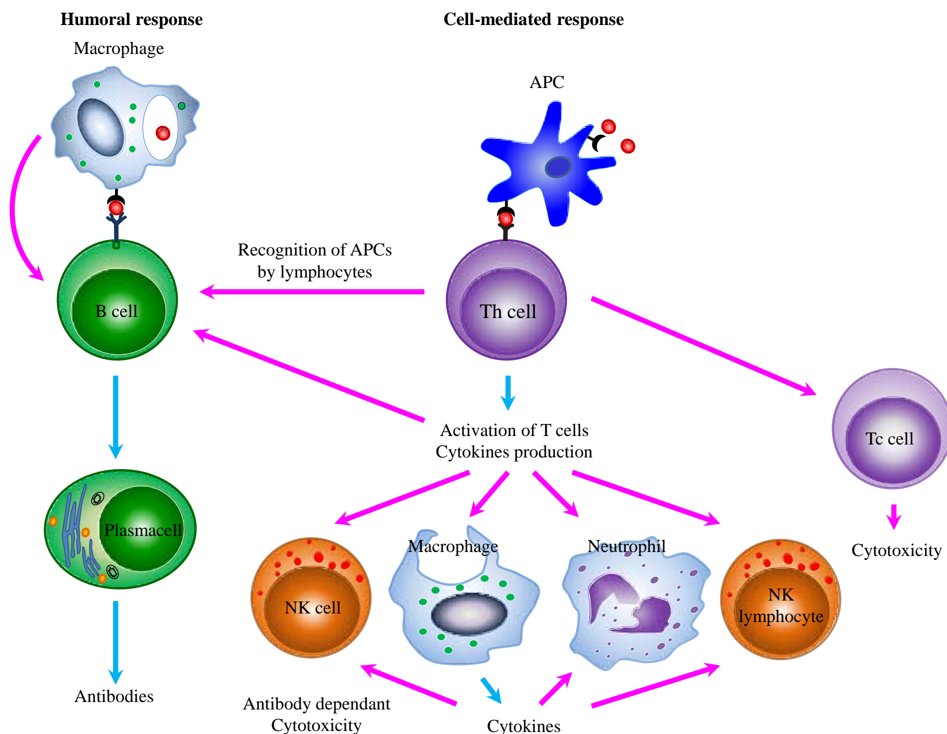


Figure 40. The specific immune response. *APC*: Antigen presenting cell (Adapted from Drouet-Viard and Fortun-Lamothe, 2002).

Antigen presentation occurs when an antigen-glycoprotein combination capable of activating T-cells appears in a cell membrane. This process is possible through an APC, whose mission is to capture, to proteolytically process inside these cells and to then present the antigen to T-cells in conjunction with MHC molecules.

The cell-to-cell interactions of the adaptive immune response are critically important in protecting against pathogens. These interactions are orchestrated by the immunological synapse whose primary components are the TCR and MHC molecules. The major function of the TCR is to recognize antigens in the correct MHC context and to transmit an excitatory signal to the cell's interior (Mayer and Nyland, 2006). The MHC is a set of neighboring genes in a single chromosome whose function is to encode those molecules that are essential for antigen recognition by T-cells and to initiate immune responses (Gomez-Lucia *et al.,* 2007). MHC molecules comprise the glycoproteins present in the membranes of most nucleated cells, among which immunocompetent cells are found. MHC molecules are easily detectable in leukocytes; for this reason they are called leukocyte antigens (LA). According to the species, MHC is given a name that generally corresponds to the initial species. The nomenclature for rabbit is RLA (Gomez-Lucia *et al.,* 2007).

2.4.2.1. Role of the Mayor Histocompatibility Complex (MHC) in immune responses

Based on their structure and function, MHC genes generally cluster into three groups: Classes I, II, III (Ujvari and Belov, 2011). Class I MHC molecules are present in most nucleated cells and are composed of a glycosylated heavy chain of 45 kDa that is non-covalently associated with the β2-microglobulin of 12 kDa (Gomez-Lucia *et al.,* 2007). Class I heavy chains are composed of three extracellular domains (α1, α2 and α3), transmembrane and cytoplasmic regions. The α3 region corresponds to the point of attachment for the T-cell co-receptor, CD8 (Gomez-Lucia *et al.,* 2007). The binding groove of Class I MHC molecules is essentially important for antigen binding and presentation on T-cells (Hassan and Ahmad, 2011). They bind specifically to varying types of ligands and cell-surface receptors in order to elicit an immune response (Hassan and Ahmad, 2011). MHC I molecules are localized in the endoplasmic reticulum where an α chain is associated with a protein known as calnexin. Subsequently, β2-microglobulin binds to the α chain by displacing calnexin. The MHC I protein acquires a conformational folding of these molecules itself and the groove is formed at the site between alloantigenic α1 and α2 (Gomez-Lucia *et al.,* 2007) (**Figure 41**).

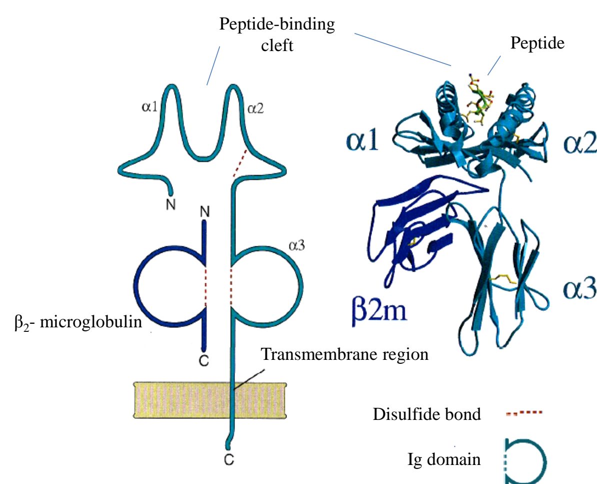


Figure 41. Structure of major histocompatibility complex class I (MHC I) (Abbas and Lichtman, 2005).

The main function on the ubiquitously expressed classical Class I α molecules is to selectively bind the short peptides deriving from proteasomal degradation of cytosolic proteins and to present these peptides on the cell surface for surveillance by $CD8^{+}$ T lymphocytes. By this mechanism, the non-self peptides deriving from intracellular pathogens can be detected by the immune system, and infected cells can be targeted for destruction (Yanover and Bradley, 2011). Non-classical MHC Class molecules (I β) accomplish a variety of cellular tasks, which are commonly performed by epithelial cells, specifically in areas of cellular transport and regulation of the lymphocyte response to altered epithelial cells and possibly bacterial antigens (Blumberg, 1998).

The MHC class II molecular structure includes heterodimers composed of an α heavy chain glycoprotein α and β light. The β2 region corresponds to the point of attachment for the T-cell co-receptor that class II-restricted CD4 corresponds to (Gomez-Lucia *et al.,* 2007). MHC Class II molecules are also classified into classical (II α) and non-classical (II β) categories, respectively based on their ability or inability to present antigens (Ujvari and Belov, 2011) (**Figure 42**). These glycoproteins present exogenously-derived antigens of $CD4^{+}$ T helper cells, triggering an immune response such as activation of antibody production by B-cells, resulting in the destruction of the invaded cell (Cresswell *et al.,* 2005).

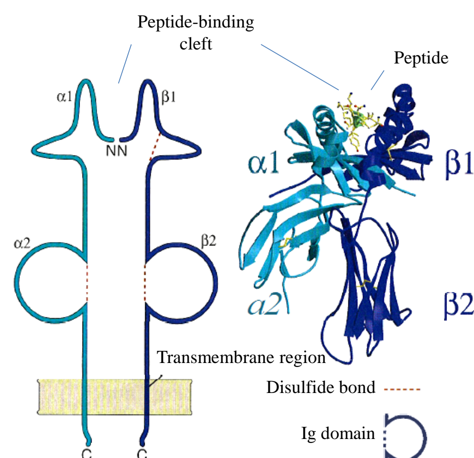


Figure 42. Structure of major histocompatibility complex class II (MHC II) (Abbas and Lichtman, 2005).

Other genes located in the MHC can be grouped into class III and encode a large number of proteins with diverse functions without antigen presenting capacity (Ujvari and Belov, 2011).

Inactive T-cells have TCR receptors that recognize Class I or Class II MHC proteins. Receptors also have binding sites that detect the presence of specific-bound antigens. If an MHC protein contains any antigen other than the specific target of a particular kind of T-cell, the T-cell remains inactive. If the MHC protein contains the antigen that the T-cell is programmed to detect, binding occurs. This process is called antigen recognition because the T-cell recognizes an appropriate target (Roitt *et al.*, 1996).

As previously cited, recognition of endogenous antigens, both viral and tumoral, follows the endogenous or cytosolic route, expressed by MHC class I (Yanover and Bradley, 2011). The

proteasome consists of two inner rings and outer two endogenous antigens, which are processed to small peptides of 8 to 10 amino acids which, in turn, are introduced into the rough endoplasmic reticulum (RER) to combine with MHC molecule I. Transport of peptides from the cytosol into the lumen of the endoplasmic reticulum is performed by the TAP transporter superfamily (transporter associated with antigen processing), a polypeptide spanning the membrane which culminates by accommodating the peptide in the groove between α1 and α2 MHC I in its final form (Murphy *et al.,* 2008).

The complex formed by the MHC class I peptide is bound to leave the RER in the form of vesicles discharged to the Golgi apparatus and then to the cell surface, where they are exposed to the recognition of cytolytic CD8 lymphocytes (Murphy *et al.,* 2008). Cytotoxic T-cells seek out and destroy abnormal and infected cells. Cytotoxic T-cells are highly mobile and roam throughout injured tissues. An inactive cytotoxic T-cell must not only encounter an appropriate antigen bound to Class I MHC proteins, but must also receive co-stimulation from the membrane which it is in contact with. It is then activated and undergoes divisions, which produce memory cells and active cells. When one of the active cells encounters a membrane displaying the target antigen, bound to the Class I MHC proteins of another cell, it uses one of several methods to destroy the cell (Roitt *et al.*, 1996) (**Figure 43**).

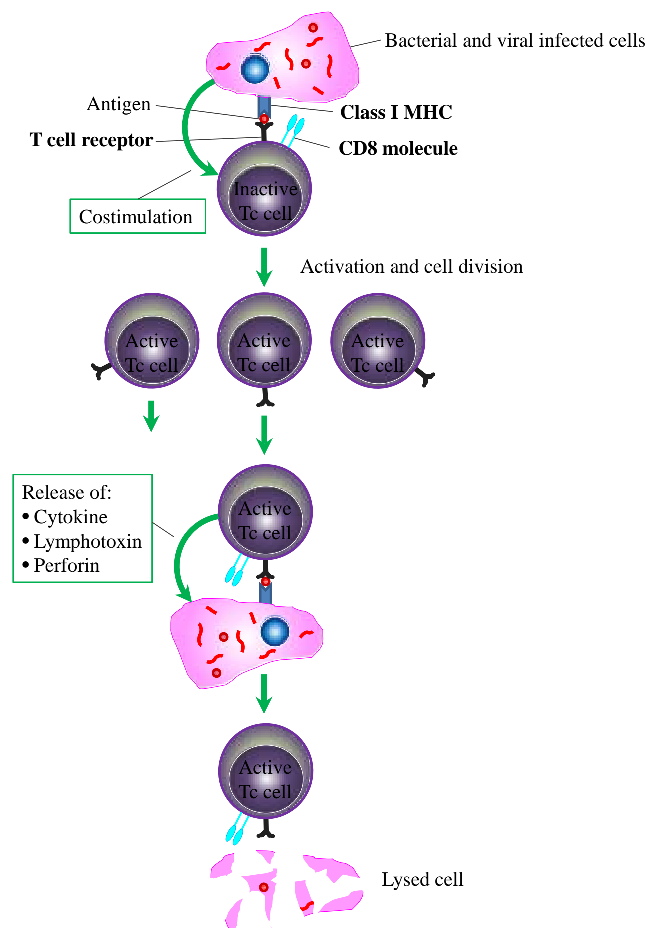


Figure 43. Activation of major histocompatibility complex class I (MHC I)**.**

Three different methods may be used to destroy the target cell. The T-cell may (1) destroy the antigenic cell membrane by releasing perforin, (2) kill the target cell by secreting a toxic

lymphotoxin, or (3) activate genes in the target cell's nucleus that promote cell apoptosis (Roitt *et al.*, 1996).

The recognition and processing of exogenous antigens are processed in endosomes, or other vesicles, specialized for processing, which are subsequently presented by MHC Class II. As with MHC I, Class II molecules are localized in the RER, where α and β chains are assembled, thereby acquiring the final constitution. In the RER, they bind to a string, called an invariant, which corresponds to CD74 (Murphy *et al.,* 2008). CD74 is an integral membrane protein, which was thought to function mainly as an MHC Class II chaperone. However, CD74 has been recently shown to play a role as an accessory-signaling molecule (Starlets *et al.,* 2006). The complexes formed by the three components are transported in the form of vesicles to the Golgi apparatus. These vesicles fuse with endosomes, which contain the peptides processed and, given the acidic pH of these structures, the invariant chain degrades with a small fragment of it remaining, known as "clip", in the groove between domains α1 and β1. Then, the clip is replaced with peptide processing to thus emerge in the cell membrane to which it is fused, while the complex formed by the MHC II remains and is anchored to exogenous antigen, which is recognized by CD4 helper lymphocytes (Murphy *et al.,* 2008).

Inactive CD4 T-cells must be exposed to the appropriate antigens bound to Class II MHC proteins. Cells then undergo activation by dividing to produce active cells and memory cells. Active cells secrete cytokines that stimulate cell-mediated and antibody-mediated immunities. They also interact with sensitized B-cells (Roitt *et al.*, 1996) (**Figure 44**).

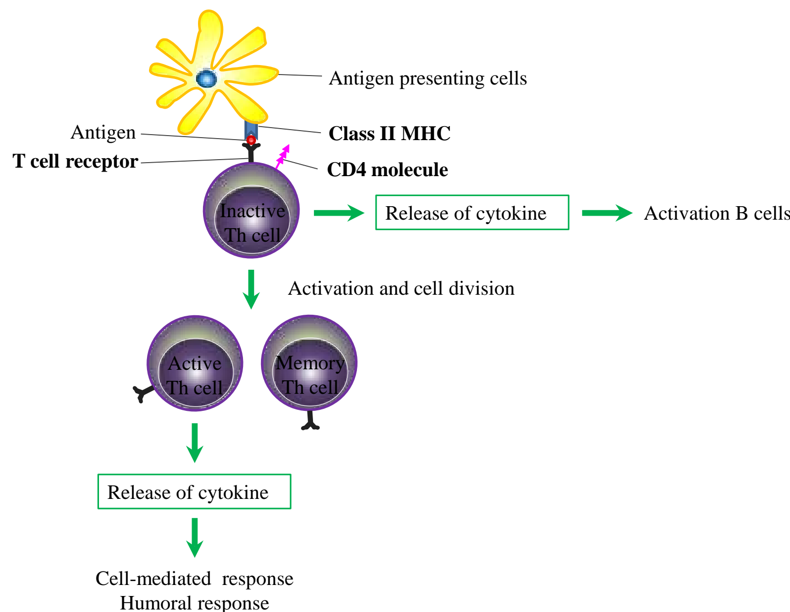


Figure 44. Activation of major histocompatibility complex class II (MHC II)**.**

While the activation of either the T helper or cytotoxic lymphocytes leads to the synthesis of lymphokines or to the lysing of cells, respectively, when B lymphocytes are activated, its primary function is immunoglobulins production.

B-cell membranes contain Class II MHC proteins. During sensitization, antigens are brought into the cell by endocytosis. Antigens subsequently appear on the surface of the B-cell bound

to Class II MHC proteins. Once this happens, the sensitized B-cell is prepared; however, it generally does not undergo activation unless it receives the appropriate stimulus from a helper T-cell (Roitt *et al.*, 1996).

The helper T-cell binds to the MHC complex, recognizes the presence of an antigen and begins to secrete cytokines that promote B-cell activation. After activation, these same cytokines stimulate B-cell division, accelerate plasma cell formation and enhance antibody production (Abbas and Lichtman, 2005).

2.4.2.2. Role of the B-cell receptors (BCR) in immune responses

Antibody maturation leads to not only the formation of B-cell receptors (BCRs) with a high affinity to antigen, but also the production of different Ig isotypes. Different antibody isotypes vary in terms of their activities (half-life, binding to Fc receptors, ability to activate the complement system) and tissue localization. Thus they are necessary for an optimal humoral response against pathogens. The molecular mechanism responsible for exchanging Ig isotypes (IgM to IgG, IgA, or IgE) and, at the same time, for retaining antigen specificity and affinity is called Ig class switch recombination (Kracker and Durandy, 2011).

Mature B lymphocytes are activated by a specific antigen-binding molecule on its membrane. The antigen receptor is a membrane-bound immunoglobulin which can recognize antigen alone (Snyder, 2012). B-cell receptors are integral membrane proteins present in thousands of identical copies exposed on the cell surface. BCR usually consists of an antigen-binding subunit (the membrane immunoglobulin, or MIg), which is composed of two IgHs (Immunoglobulin Heavy Chains) and two IgLs (Immunoglobulin Light Chains), and a signaling subunit, which is a disulfide-linked heterodimer of Igα (CD79A) and Igβ (CD79B) proteins. During B-cell differentiation (and long before any possible encounter with an antigen), the DNA in this locus is cut and recombined to make an intact gene for the heavy chain. Since MIg is always associated with the Igα/Igβ heterodimer to collectively form the B-Cell receptor complex (BCR), two molecules of this heterodimer associate with one MIg to form a single BCR. The Igα/Igβ heterodimer carries out the signal transducing function of the complex (Rheingold *et al.,* 2003) (**Figure 45**).

BCRs have a unique binding site called an antigenic determinant or epitope. Non covalent binding depends on the complementarity of surfaces of both the receptor and the epitope. In the absence of a specific antigen, mature B-cells survive in peripheral circulation for only a few days, after which they undergo apoptosis to maintain B lymphocytes circulation optimal in peripheral circulation. When the receptor is on the cell surface of B lymphocytes, it functions to transmit intracellular signals which regulate cell growth and differentiation, and it binds to an antigen to generate the immune response (Calame, 2001). Most B-cell antigens are T-dependent. In other words, and as seen in the previous section on the MHC, the B-cell needs to be in direct contact with Th lymphocytes and Th lymphocyte cytokines in order to be fully activated. There are some T-independent antigens, and one of the best-known examples of a T-independent antigen is LPS (Lipopolysaccharide) bacteria. At low concentrations, LPS stimulates the production of specific antibodies (LPS-specific), but it can cause the polyclonal activation of B-cells at high concentrations, regardless of their antigen specificity. Bacterial cell wall polysaccharides and bacterial flagellin can also serve as T-independent antigens. Cell wall polysaccharides are characterized by possessing repetitive monosaccharide subunits, while bacterial flagellin is a repetitive polymeric protein

(Rojas-Ramos, 2003). The signal initiated by antigen binding to the B-cell receptor complex causes B-cell growth and proliferation, and the creation of an amplified clone of effector cells secreting antigen-specific immunoglobulin. Activation of the B-cell receptor by an antigen also results in the production of memory cells that persist in circulation to produce a more rapid immune response after future challenges by the same antigen. Bound antigen molecules are engulfed into the B-cell by receptor-mediated endocytosis. The antigen is digested into fragments, which are then displayed on the cell surface nestled inside a Class II histocompatibility molecule (Silver and Cornall, 2003).

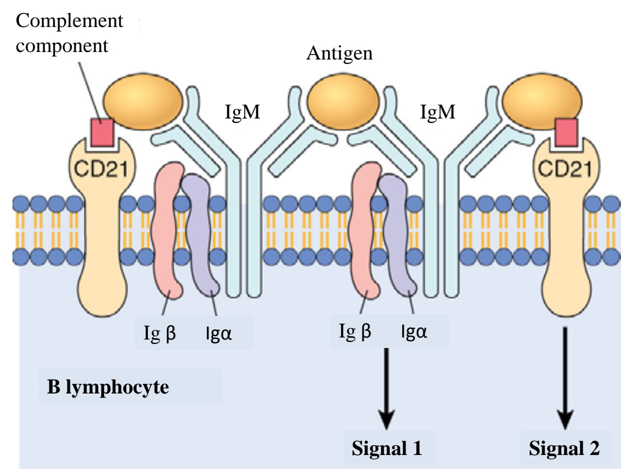


Figure 45. The B lymphocyte antigen receptor complex. Membrane IgM and signalling molecules Igα and Igβ. CD21, also known as complement receptor-2, binds complement components and activates B lymphocytes. *Ig*: Immunoglobulin (Snyder, 2012).

2.4.2.3. Role of the T-cell receptors (TCR) in immune responses

A key step of the immune response is the detection by T lymphocytes, due to their T-cell receptor (TCR), of foreign peptides bound to major histocompatibility complex molecules on the surface of APCs (Puech *et al.,* 2011). The TCR interacts with the peptide-major histocompatibility complex (pMHC) to enable T-cell development and to trigger adaptive immune responses. For this reason, TCR:pMHC interactions have been intensely studied for over two decades (Edwards *et al.,* 2012).

In addition to its prominent physiological importance, interaction of the TCR with pMHC arouses enormous interest owing to a number of features.

- ✓ Recognition is specific since T lymphocytes have been reported to detect a single cognate pMHC complex on APCs by exposing many tens of millions of proteins on their membranes (Irvine *et al.,* 2002). The TCR can structurally recognize different MHC-bound peptides, yet can also discriminate between peptides and MHCs, which differ by a change as slight as a single polymorphism (Reboul *et al.,* 2012).

- ✓ The TCR repertoire is sufficiently rich to cope with many millions of potentially harmful structures and is specific enough to avoid autoimmune phenomena.

- ✓ Recognition and subsequent activation is sufficiently rapid to occur during a typical contact, whose duration ranges from seconds to minutes between an APC and a T lymphocyte (Miller *et al.,* 2004).

- ✓ Recognition may generate vastly different outcomes, ranging from full lymphocyte activation to anergy, following minute variations in the peptide antigen sequence (Sloan-Lancaster *et al.,* 1996).

During maturation, intrathymic TCR-specific receptor acquisition occurs (Abbas and Lichtman, 2005). Individual lymphocytes' antigen specificity is attributed to their respective TCR, which is genetically determined. TCRs are classified as either αβ-TCR or γδ-TCR based on the composition of their disulfide-liked heterodimers (Snyder, 2012).

The TCR is a heterodimer composed of one α and one β chain of approximately equal length. Each chain has a short cytoplasmic tail, but it is too short to be able to transduce an activation signal to the cell. Both chains have a transmembrane region comprising hydrophobic amino acids by which the molecule is anchored on the cell membrane. Both chains have a constant region and a variable region similar to the immunoglobulin chains. The variable region of both chains contains hypervariable regions that determine specificity for an antigen (Mayer and Nyland, 2006) (**Figure 46a**). Individual αβ-TCRs are covalently linked to a cluster of five polypeptide chains: three comprising the CD3 molecule and two comprising the β-chain. Both TCRs are associated with CD3, and together they form the TCR-CD3 complex, which is essential for the transmission of antigen recognition signal transduction (Snyder, 2012).

Unlike membrane-bound immunoglobulin on B lymphocytes, the αβ-TCR can only recognize antigen after it has been processed into peptide fragments and associated with MHC molecules, while γδ-TCR can recognize native antigen in the absence of MHC binding, and they do not rely exclusively on the δ-chain as a signal transducer.

Although all T lymphocytes express the TCR-CD3 complex, the CD8 and CD4 accessory molecules required to help stabilize the interaction between TCR and MHC I and II respectively, are commonly used as markers to classify subsets of thymocytes, which allows them to classified into three groups: double-negative ($CD4^{-}CD8^{-}$), double-positive ($CD4^{+}CD8^{+}$) and positivity ($CD4^{+}CD8^{-}$/ $CD4^{-}CD8^{+}$). Other accessory molecules expressed by T lymphocytes include CD2, integrins and CD28. T lymphocytes' activation is provided by the binding between TCR or CD4/CD8 with the MHC- antigen complex or, in another case, by accessory molecule CD28 with its ligands B7-1 (CD80) and B7-2 (CD81) expressed in activated dendritic cells, B lymphocytes and macrophages (**Figure 46b**) (Snyder, 2012).

These adhesion molecules are responsible for Ag activation being carried out between presenting cells and target cells. Their expression can increase in response to cytokines. The multiple interactions of the TCR with Ag/MHC, and between the accessory and co-stimulatory molecules with their ligands have been termed "immunological synapse". Consequently, it triggers a cascade of biochemical reactions in the cytoplasm of the T-cell, thus leading to activation, proliferation and differentiation. Th-cell activation is the core of the cellular response which, in turn, acts on macrophages, NK cells and Tc lymphocytes to then acquire the ability to lyse those cells carrying antigen-induced activation (Mayer and Nyland, 2006).

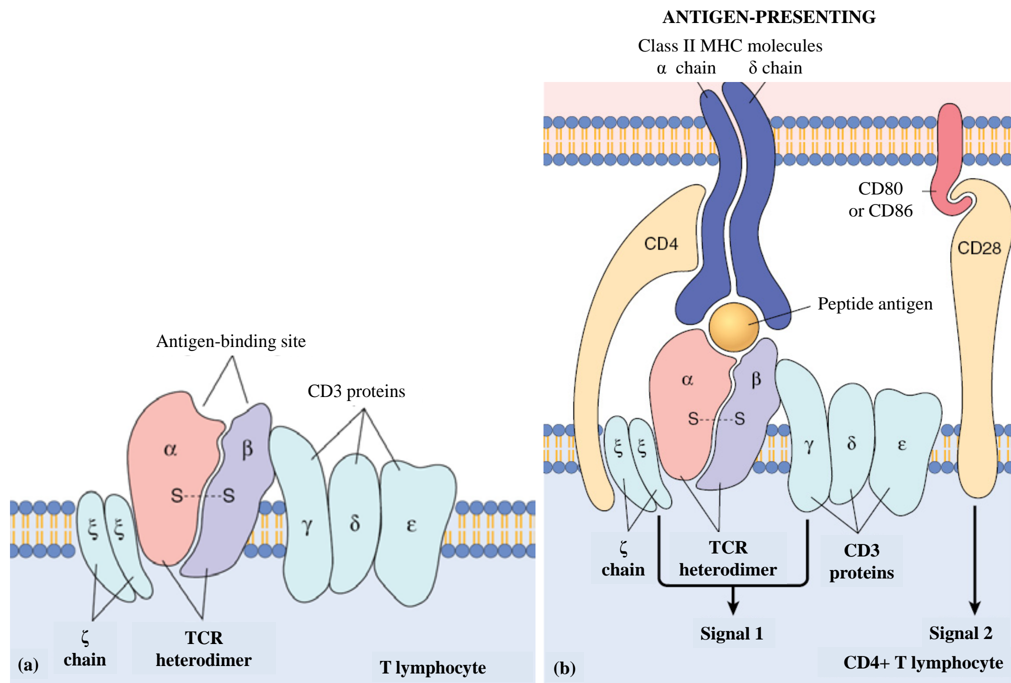


Figure 46. The T lymphocyte receptor complex (T lymphocyte receptor [TCR]). (**a**) α-TCR and β-TCR chains complexed with CD3 γ-, δ-, and ε-chains and the invariant, ζ-chains (Kumar *et al.,* 2005) (**b**) Illustrating how the TCR recognises an antigen in the major histocompatibility complex context on the antigen-presenting cell (top) and how the ζ-chains and CD3 γ-, δ-, and ζ-chains deliver one of the two required signals for T lymphocyte activation. The second required signal is delivered by the co-stimulator molecules CD28 on the T lymphocyte and B_7 on the antigen-presenting cell. *MHC,* Major histocompatibility complex (Kumar *et al.,* 2009b).

2.4.2.4. Cell-mediated response

Cellular immunity, along with humoral immunity, comprises the adaptive immune response. Unlike humoral immunity, which involves B-cells and antibodies, cellular immunity responds to an antigen by the activation of macrophages, cytotoxic T-cells, CD4 T-cells, natural killer (NK) cells, and to the release of cytokines (Janeway *et al.,* 2005). When T lymphocytes are activated by antigens and receive appropriate co-stimulatory signals, they clonally expand as a result of their IL-2 secretion. This clonally expanded population of T lymphocytes, of the same antigen specificity, differentiates into populations of effector and memory lymphocytes (Snyder, 2012). After T helper lymphocytes recognize a specific antigen bound to the MHC class II molecules presented by an APC, they can initiate several key immune processes (Roitt *et al.,* 1996), including:

- ✓ Directing the fine specificity of the response (i.e., by determining which antigens and which epitopes are recognized).
- ✓ Selecting appropriate effector mechanisms (i.e., B-cell activation or Tc generation) to be directed against target pathogens. The three most easily recognizable patterns of an effector mechanism that can be selected are: $CD8^+$ cytotoxic T-cells, antibody plus mast cells and eosinophils, macrophage activation and delayed hypersensitivity.
- ✓ Assisting the proliferation of appropriate effector cells.

✓ Enhancing the functional activities of other cells (i.e., granulocytes, macrophages, NK cells).

Local patterns of cytokine and hormone expression help to select the effector mechanism to be activated. The initial cytokine triggering pattern by the pathogen, and the local concentration of several steroid and vitamin D_3 metabolites in lymphoid tissue, determine whether the Th-cells that develop are of the Th1 or Th2 subset (Roitt *et al.,* 1996).

Recent advances made in stem cell research have redefined the previous concept of hematopoietic hierarchy, lineage commitment and cell fate (Mucida and Cheroute, 2010). In fact, the number of defined Th-cell subsets has grown and the once rigid division of labor among them has been blurred by reports of plasticity among the subsets (Zygmunt and Veldhoen, 2011).

Actually, there are different subpopulations of Th-cells, such as Th1, Th2, Th9, Th17, Tfh cells and memory T-cells. When naive Th0 cells encounter an antigen in secondary lymphoid tissues, they are capable of differentiating into inflammatory Th1 cells, helper Th2 cells or pathogenic Th17 cells, which are distinguished by the cytokines they produce (**Figure 33).**

Another functionally distinct subpopulation of $CD4^+$ T lymphocytes is the regulatory T lymphocytes (Treg), which express CD25 on their cell surface. Their main function is to suppress the response of the self-reactive CD4 lymphocytes that have escaped the negative selection process in the thymus. After activation through FoxP3 transcriptional repressor, this subpopulation of lymphocytes produces immunosuppressive and anti-inflammatory cytokines IL-4, IL-10 and TGF-β. They have been shown to play a role as suppressor lymphocytes of immunity and inflammation (**Figure 47**).

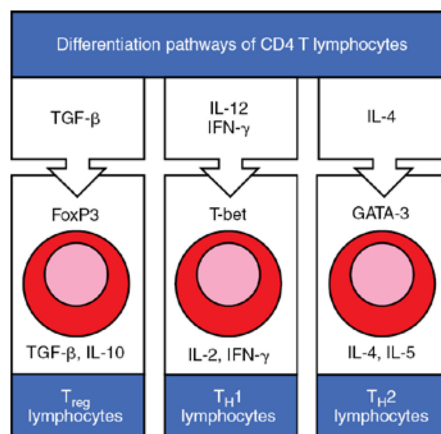


Figure 47. Differentiation and expression of CD4 T lymphocytes. Cytokines in the extracellular tissue fluids direct the differentiation of CD4 T lymphocytes to specific secretory types, so that they secrete specific cytokines that perform specific functions on other lymphocytes in the environment. *FoxP3*: Forkhead box P3; *IFN*: Interferon; *IL*: Interleukin; *TGF*: Tumor growth factor (Parham, 2009).

It has been reported that Th17 cells (designated as such by their production of IL-17) are able to differentiate (in humans) in response to IL-1, IL-6, IL-23 and TGF-β. IL-17 enhances the severity of some autoimmune diseases. Furthermore, it induces the recruitment of monocytes and neutrophils to inflammation sites (Snyder, 2012).

It is equally important to note that one subpopulation can exert inhibitory influences on another. IFN-γ, produced by Th1 cells, inhibits the proliferation of Th2 cells and the differentiation of Th17 cells, while IL-10 produced by Th2 cells inhibits the production of IFN-γ by Th1 cells. In addition, IL-4 inhibits the production of Th1 cells and the differentiation of Th17 cells. Thus, the immune response is directed to the type of response required to deal with the pathogen encountered: cell-mediated responses for intracellular pathogens or antibody responses for extracellular pathogens (Mayer and Nyland, 2006).

2.4.2.5. Humoral response

As mentioned above, besides recognizing antigens or infected cells, CD4 Th2 cells also activate B-cells and the humoral immune response (Janeway *et al.,* 2005).

Humoral immunity, which is the branch of the immune system governed by B-cells, provides robust protection against extracellular pathogens through the secretion of immunoglobulins that neutralize or facilitate the destruction of microorganisms (Montgomery *et al.,* 2011; Zaheen and Martin, 2011) (**Figure 48**).

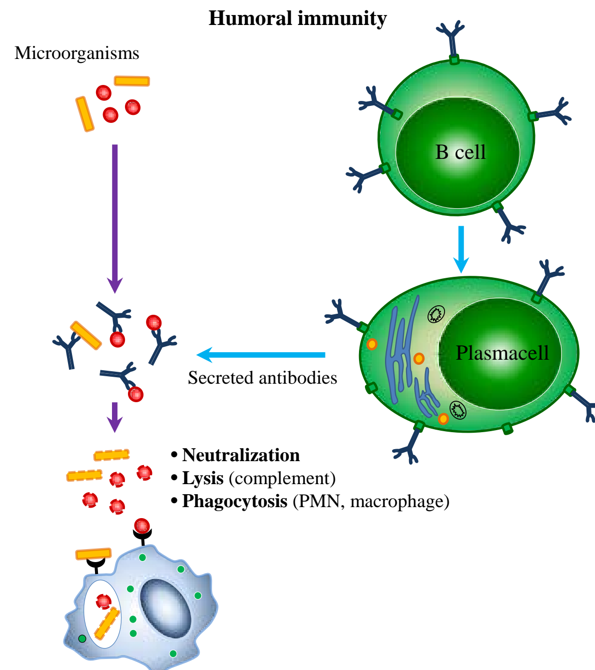


Figure 48. Schematic representation of the humoral immunity mediated by the soluble antibody proteins produced by B lymphocytes. Antibodies participate in immunity either by directly neutralizing extracellular microbes or activating complement and certain effector cells (polymorphonuclear neutrophils [PMNs] and macrophages) to kill microorganisms.

The nature of the antigen itself can dictate which B-cell subset is recruited into an antibody response. Using model antigens in rodents, B-cell antigens have been classified as either T-cell independent (TI) or T-cell dependent (TD). TI antigens promote B-cell proliferation, differentiation and antibody production in the absence of T-cells, and are further classified into two subgroups: type 1 (TI-1) and type 2 (TI-2). TI-1 antigens are mitogens that stimulate all B-cells to produce an antibody in a polyclonal manner, irrespectively of antigen specificity (Swanson *et al.,* 2010).

The first stage in the humoral pathway of immunity is the ingestion (phagocytosis) of foreign matter by macrophages or, in general, by APC. These cells containing digestive enzymes digest the infectious agent and then display some of its components on their surfaces to be combined with MHC II proteins. Helper T-cells ($CD4^+$) recognize this presentation and bind to the MHC class II-antigen peptide complex. This binding triggers the APC to release cytokine IL-1, which activates the helper T-cell and multiplies rapidly. Thus, each helper produced T-cell has a receptor specific for the original processed antigen; this is called the activation phase.

The next phase, known as the effector phase, involves communication between helper T-cells and B-cells (Roitt *et al.,* 1996). This phase begins when a B-cell exhibits an IgM receptor specific for the same antigen originally engulfed by the APC on its surface, and then encounters and binds the antigen. The B-cell engulfs the complex by receptor-mediated endocytosis; therefore, the antigen is ready to be processed (Pier *et al.,* 2004). Inside the B-cell, the antigen binds class II MHC molecules and is displayed on the surface. Helper T-cells bind to the antigen displayed on the surface of the B-cell causing the helper T-cell to release cytokines that stimulate the B-cell to divide and proliferate into identical B-cell copies. B-cell descendants become either plasma cells or B memory cells.

Plasma cells begin to manufacture huge quantities of antibodies with an identical specificity to that of the surface receptors on the parent B-cell, which bind to the foreign invader (the antigen) and prime it for destruction. Simultaneously, the complement system is signaled to attack and puncture holes in bacteria (Janeway *et al.,* 2001). B memory cells retain a "memory" of the specific antigen that can be used to faster mobilize the immune system if the body encounters the antigen later in life. These cells generally persist for years (Roitt *et al.,* 1996).

3. EFFECT OF ENVIRONMENT ON THE RABBIT IMMUNE SYSTEM

It is well-known that rabbit health is considered a main handicap on commercial farms. This, knowledge of rabbit immune response is important to determine how animals react to different sporadic situations of stress or challenge that are present commonly on commercial farms, such as heat stress, chronic timely interaction with pathogens and high-demanding reproductive conditions.

As previously described, different immune response types fall into two main categories: innate (or non adaptive) immune responses and adaptive immune responses (Roitt *et al.,* 1996).

The specific immune defense mechanism has better evolved than innate immunity, and can recognize and react to numerous infectious and non infectious agents through lymphocytes among other cells (Gonzalez *et al.,* 2011). In fact, adaptive immunity generally consists in cell-mediated immunity mediated by T lymphocytes and humoral immunity mediated by B lymphocytes (Synder, 2012). In particular, T-cells play a key role in the initiation, maintenance and control of the immune response. To start the specific immune response, antigen recognition by lymphocytes and the subsequent activation thereof are required.

Indeed, different subpopulations of T lymphocytes have been recognized, and each plays a very important role in the rabbit immune response. Thus, it is interesting to know how these cellular populations, fundamental in the development of immune responses, modulate to face any infectious or non infectious challenges.

In this sense, there are many studies on blood parameters and lymphocyte subsets in different rabbit types (conventional and pathogen-free, neonates, pubertal and adult primiparous) (Wells *et al.,* 1999; Jeklova *et al.,* 2007a,b; Çetin *et al.,* 2009; Jeklova *et al.,* 2009), although very few data on modifications in peripheral blood lymphocyte subpopulations are available (Jeklova *et al.,* 2007a), especially in industrial rabbits under field conditions.

Another problem faced by immunology studies in rabbits is the limited number of available monoclonal antibodies (Drouet-Viard and Fortun-Lamothe, 2002).

A complete blood analysis is a good indicator of general health as stress and numerous illnesses can modify hematological parameters, especially with regard to erythrocyte and lymphocyte counts (Hinton *et al.,* 1982). Thus, blood tests offer the chance to investigate the presence of several metabolites and other constituents, and can help detect stress conditions, which can be nutritional, environmental or physical in nature (Aderemi, 2004). Physiological parameters (hormones, heart rate, immune reactions), when considered in relation with other parameters (behavior, morbidity), can be used as a welfare indicator (Hoy and Verga, 2007). However, when considering the stipulated oscillations of hematological parameters, it is difficult to interpret altered results (Poljičak-Milas *et al.,* 2009).

It is known that in rabbits, as in other animal species, hematological and biochemical parameters are significantly influenced by the strain of rabbits (Laird *et al.,* 1970), gender (Laird *et al.,* 1970; Wolford *et al.,* 1986; Çetin *et al.,* 2009), season (McLaughlin and Fish, 1994) and daily variation (Fox and Laird, 1970), with the lowest values appearing early afternoon and evening. Physiological conditions include age (Jeklova *et al.,* 2007a, 2009; Poljičak-Milas *et al.,* 2009), pregnancy (Wells *et al.,* 1999; Kim *et al.,* 2002b; Çetin *et al.,*

2009), nutrition (Schermer, 1967; Franci *et al.,* 1996a), environment and daily variations in reproductive rhythm (Fox and Laird, 1970; Bartolotti *et al.,* 1989; Harcourt-Brown, 2002b; Campbell, 2004).

Accordingly, quite a wide physiological leukocyte count range exists in rabbits, from 5 to 12.5 $\times$ 10^9/L (Kabata *et al.,* 1991; Carpenter, 2005; Jenkins, 2006). The ranges proposed for the share of specific forms of leukocytes are also ample: 30-80% for lymphocytes, and 20-75% for heterophils. The values of both eosinophils and monocytes are lower: 0-4%. The range of basophils share is somewhat wider: 0-7% (Kabata *et al.,* 1991; Jenkins, 2006). Hematological parameters and differential leukocyte counts in the blood of rabbits are included in the **Tables 5** and **6** by Poljičak-Milas *et al.* (2009).

Table 5. Hematological parameters in rabbits. *MCH:* Mean corpuscular hemoglobin; *MCHC:* Mean corpuscular hemoglobin concentration; *MCV:* Mean corpuscular volume; *PCV:* Packed cell volume; *RDW:* Red cell distribution width; *SD:* Standard deviation (Poljičak-Milas *et al.,* 2009).

	Male rabbits (n=12)		Female rabbits (n=14)	
Hematological parameter	Range	Mean ± SD	Range	Mean ± SD
Leukocytes (10^9/L)	4.2 - 12.3	8.33 ± 2.48	4.4 - 13.2	8.81 ± 3.03
Erythrocytes (10^{12}/L)	4.08 - 6.96	5.86 ± 0.79	4.89 - 6.85	6.21 ± 0.52
Haemoglobin (g/L)	104 - 140	127.5 ± 11.99	123 - 151	134.86 ± 8.53*
PCV (L/L)	0.29 - 0.44	0.38 ± 0.04	0.36 - 0.44	0.41 ± 0.03
MCV (fL)	61.4 - 70.3	65.56 ± 3.14	60.2 - 72.8	65.66 ± 2.91
MCH (pg)	19.7 - 26	21.93 ± 1.91	19.2 - 25.2	21.79 ± 1.36
MCHC (g/L)	309 - 371	334.33 ± 16.01	316 - 346	331.78 ± 8.33
RDW	15.3 - 17.2	16.09 ± 0.55	14 - 16.4	15.53 ± 0.65**
Thrombocytes (10^9/L)	390 - 821	529.75 ± 125.85	353 - 703	499.93 ± 94.0

Values are expressed as mean value ± standard deviation. Significance of differences with regard to gender at the level of: *$P<0.05$; **$P<0.01$.

Table 6. Differential leukocyte counts in the blood of rabbits. *SD*: Standard deviation (Poljičak-Milas *et al.,* 2009).

	Male rabbits (n=12)		Female rabbits (n=14)	
Blood cells	Range	Mean ± SD	Range	Mean ± SD
Lymphocytes (%)	16 - 70	38.67 ± 19.55	20 - 69	46.14 ± 18.52
Monocytes (%)	0 - 3	0.75 ± 1.21	0 - 3	0.5 ± 0.94
Heterophils (%)	27 - 94	60.25 ± 20.52	27 - 79	53 ± 18.51
Eosinophils (%)	0 - 2	0.25 ± 0.62	0 - 3	0.21 ± 0.8
Basophils (%)	0 - 1	0.08 ± 0.29	0 - 1	0.14 ± 0.36

Values are expressed as mean value ± standard deviation.

Aberrations in the rabbit leukogram can be more difficult to interpret than those in most domestic animals. Rabbits do not commonly develop leukocytosis with bacterial infections, but may display an inverse heterophil/lymphocyte (H:L) ratio, which is secondary to any source of stress (cortisol), including stress from transport or chronic disease (Harcourt-Brown, 2002). Venipuncture and blood collection processes do not appear to have such effects (Harcourt-Brown, 2002). Stress (endogenous cortisol release) should not be confused with excitement (epinephrine release), and excitement should actually cause lymphocytosis, while stress may result in lymphopenia (Lester *et al.,* 2005). The presence of other systemic signs of illness, such as fever or toxic changes, may help determine if leukogram changes are

caused by infection. Given the cell function similarity across species, other changes in the leukogram may have the same general etiologies. Leukocytosis can occur with lymphosarcoma, especially if abnormal lymphocytes are present in the stained blood smear. Leukopenia, particularly lymphopenia, may indicate chronic disease. Chronic parasitism may cause an eosinophilia (Benson and Paul-Murphy, 1999). Monocytosis, if present, suggests chronic inflammation.

Rabbit diseases, stress and administration of cortical steroids (Jeklova *et al.,* 2008) rarely lead to increased total leukocyte counts; however in some cases, stress has been described to possibly increase total leukocyte counts by 15-30% (Campbell, 2004). Conversely, they more frequently cause a change in the share of specific leukocyte forms given their re-distribution (Toth and Krueger, 1989; Jenkins, 2006).

The effect of age on lymphocyte subset distribution in mammals and humans has been described, but with conflicting results in relation to the changes in T lymphocytes being reported. Several authors did not confirm any changes, decreases, and even increases, in the percentages of these lymphocyte subsets in different compartments (cats: Wilson *et al.,* 1996; calves: Sellon *et al.,* 1996; cows: Ohtsuka *et al.,* 2009; dogs: Faldyna *et al.,* 2005; pigs: Joling *et al.,* 1994; Borghetti *et al.,* 2005; Brown *et al.,* 2006 and humans: Erkeller-Yuksel *et al.,* 1992; Hulstaert *et al.,* 1994; Globerson and Effros, 2000). However, Stepanova *et al.* (2007) reported postnatal changes in T lymphocyte subsets in pigs, especially γδ T lymphocytes, in blood, spleen and lymph nodes. During ontogeny, the frequency of $CD8^+CD3^+CD4^-$ and γδ-TCR significantly increased, but was compensated by a reduced proportion of $CD4^+$ lymphocytes. The relative counts detected in this study were reported by other authors in the same species (Joiling *et al.,* 1994; Sinkora *et al.,* 1998; Solano-Aguilar *et al.,* 2001). Significant changes with age have been demonstrated in neonatal pigs, including increases in B-cells and T-cells, and a bigger proportion of total T-cells expressing MHC II (Grierson *et al.,* 2007). Sellon *et al.* (1996) described age-related changes in lymphocyte subsets in perinatal cats, while Wilson *et al.* (1996) reported T-cell subsets in blood and lymphoid tissues obtained from fetal calves, maturing calves and adult cattle. Postnatal development of lymphocyte subpopulations in blood and lymphoid organs in dogs has also been characterized (Faldyna *et al.,* 1998, 2001, 2005), showing that the percentage of B lymphocytes in peripheral blood lowers with advancing age, while $CD8^+$ increases. In humans, however, there are reports of changes in the lymphocyte subset in aged persons (Erkeller-Yuksel *et al.,* 1992; Hulstaert *et al.,* 1994; Franceschi *et al.,* 1995; İkincioğulları *et al.,* 2004). In addition, Globerson and Effros, (2000) described how the frequency of naive T-cells diminishes from birth to adulthood, and how their functional capacity declines. Moreover, total leukocyte counts and their distribution vary according to the age of rabbits. The highest total leukocyte count was recorded in 3-month-old rabbits, and later in 1-year-old mature rabbits (Poljičak-Milas *et al.,* 2009). After the age of 13 months, heterophils and lymphocytes may be present in approximately equal numbers (Campbell, 2004). The first spike was the lymphocyte count growth results, while the other was the heterophils count growth results (Harcourt-Brown, 2002). Similarly to other animal species and in humans, the number of total lymphocytes in weaned rabbits is lower than during breeding because of the gradual increase due to the maturation of young rabbits' immune systems (Jeklova *et al.,* 2007a, b; 2009).

Age-related variations in the numbers of T and B lymphocytes in thymus, bone marrow, spleen, popliteal lymph nodes, appendix and peripheral blood of rabbits have been described

(Fujiwara *et al.,* 1974). Recently, Archetti *et al.* (2008) found significant differences according to rabbit age in terms of hematological and biochemical parameters. These authors demonstrated how the lymphocytes count increases with age, while their percentage lowers. Jeklova *et al.* (2007a) reported prenatal and postnatal changes of lymphocyte subpopulations in peripheral blood and lymphoid organs. Smaller numbers of T-cells were detected in both peripheral blood and spleen than in the mesenteric lymph nodes of neonate SPF rabbits. In blood, all the studied lymphocyte subsets ($CD4^+$, $CD8^+$, pT^+, $CD79\alpha^+$) significantly increased during maturation due to a significant rise in absolute lymphocyte numbers. In peripheral blood, relative values of the pT^-, $CD4^-$, $CD8^-$, $CD79\alpha^-$ populations of lymphocytes lowered with the age. More recently, Jeklova *et al.* (2009) reported that lymphocytes remained at low levels at the time of birth, but sharply increased in terms of both percentages and total counts during the first month of life. When compared with adult SPF rabbits (20-week-old), the percentage values of lymphocytes were significantly lower in 1-day and 2-week-old rabbits.

In adult rabbits (2-2.5 years old), significant differences in total lymphocyte numbers have been reported, depending on gender and physiological status (Çetin *et al.,* 2009): males (5700×10^6/L), non pregnant females (4300×10^6/L) and pregnant females (2800×10^6/L). Nonetheless, the effect of management, high temperatures and LPS inoculation on the rabbit immune system have yet to be clearly described.

3.1. Effect of reproductive effort

3.1.1. Effect of reproductive rhythm on female performance and body condition

In recent years, rabbit meat production systems in Western Europe have evolved toward increasingly intensified production (Rosell, 1996). This intensification has been associated with an increased density of farm animals, which facilitates the spread of pathogens from one rabbit to another (Hermans *et al.,* 2003). In any case, rabbit susceptibility to diseases is similar to other intensively farmed animals, such as pigs, and with a similar on-farm mortality risk (Lebas, 2000; Rosell and de la Fuente, 2009).

Digestive diseases are the main cause of morbidity and mortality in growing rabbits, and they are responsible for severe economic losses among industrial rabbitries (Marlier *et al.,* 2003). Rosell and de la Fuente (2009) reported that the second cause of mortality or disease in rabbit does was digestive perturbances, which followed the pathological processes of the respiratory tract. The European ban on antibiotic type growth promoters in animal feeds has even complicated farmers' weaning management because of the widespread incidence of emerging animal diseases. Since the first outbreaks in 1997, Epizootic Rabbit Enteropathy (ERE) has threatened this industry as it can cause mortality at between 20% and 70%, and up to 100% morbidity on European rabbit commercial farms (de Blas *et al.,* 2012). ERE is characterized by abdominal distensions, emission of small quantities of watery droppings which follow drastically reduced feed intake, and is highly contagious (Licois *et al.,* 1998). Normally, this disease affects mainly young rabbits after weaning (Licois *et al.,* 2005). The etiology of this disease remains unknown; nevertheless, proliferation of *Clostridium perfringens* could be the result of ERE and could be associated with high mortality caused by this disease (de Blas *et al.,* 2012).

Although various antibiotics have proved effective against digestive diseases, it is necessary to find alternative solutions for their control in order to comply with current EU legislation. Among them, the nutritional management and different protocols of weaning have become a priority given their importance in digestive diseases. For commercial rabbit production, it has been reported that good management improves the health and productivity of the entire herd as it not only lowers high replacement rates, but also improves economic viability (Marai *et al.,* 2010). Among the management aspects, the interval between successive inseminations plays a key role (Castellini *et al.,* 2006). Piles *et al.* (2006) reported that a 10-day increase in the conception interval increased the risk of culling by up 36%; thus, the effect of insemination could affect the risk of culling in rabbit does.

In addition, some farmers are modifying management on their farms by promoting prolonged rhythm lactations. Common weaning protocols at 28-32 days postpartum (dpp) (with inseminations at 11-13 dpp) are being replaced with late weaning, after 35 dpp (with inseminations on 18-25 dpp). Currently, this is a late weaning empirical practice to reduce mortality during fattening, probably because of the protective role of milk (Fortun-Lamothe and Boullier, 2007; Gallois *et al.,* 2007), and later weaned rabbits present a more mature immune system. However, a prolonged lactation period could affect rabbit does' body condition, although knowledge on its possible effect on rabbits is scarce (Pascual *et al.,* 2006).

During rabbit breeding, the general aim is to gain greater litter sizes with good growth rates. In this way, industrial breeds have significantly improved by devising breeding genetic programmes for such criteria.

The rabbit is an animal of induced ovulation. This makes it very difficult to define age of puberty, which depends mainly on breed (small are more precocious) and body development, and rabbits approach puberty when they reach 70-75% of adult weight. Having induced ovulation, it is necessary to inoculate follicle stimulating hormones 48-72 h prior to artificial insemination (natural mating has virtually ceased). This provides maturation with a new wave of follicles (Rosell *et al.,* 2000a). Timing insemination depends on the farmer’s choice of reproductive rhythm. The most frequently used reproductive rhythms are:

- ✓ Traditional (extensive): weaning over 35-42 days, mating 21-25 days post-partum.
- ✓ Semi-intensive: weaning at 28-35 days, mating 10-12 days post-partum.
- ✓ Intensive: weaning at 21-28 days, mating 1-4 days post-partum.

On European commercial farms, the most common reproductive rhythm is based on not only the artificial insemination (AI) of does at around 11 days postpartum (dpp), but also on the weaning of young rabbits at 28-30 days of age. This protocol is well-adapted to cycle production, but it does not take into account the reproductive physiology of does (Castellini *et al.,* 2003a). Previous studies have shown that intensive reproductive rhythms increase doe’s annual production and achieve more than 11 deliveries per year (Maertens and Okerman, 1988; Cervera *et al.,* 1993); however, they reduce litter size, fertility rates and length of reproductive activity (Parigi-Bini *et al.,* 1989). It has been shown that this effect is due to the nutritional wear of doe, where gestation overlaps lactation relentlessly (de Blas *et al.,* 2003).

Semi-intensive rhythms have provided intermediate results (Castellini *et al.,* 2003b), whereas the long-term effect of extensive rhythm improves does' body condition and prolongs reproductive life (Dal Bosco *et al.,* 2003).

In Spain, rabbits on commercial farms are usually weaned at 35 days of age. A younger weaning age would help reduce the interval between partums and would lower the risk of transmitting infections from does to young rabbits. However, early weaning at 25 days of age lowers growth rates (Rodriguez *et al.,* 1981) and increased diarrhea incidence (Lebas, 1993) when young rabbits are fed commercial fattening diets. These findings can be related with low nutrient intake and insufficient development of digestive and absorptive capability during the post-weaning period (De Blas *et al.,* 1999b). On the other hand, Xiccato *et al.* (2003) reported that the early weaning of litters, even at 21 days of age, can be successfully performed without compromising kits viability and performance during the subsequent growth period. The literature available on early weaning reports different feeding and management strategies depending on weaning age. Earlier weaning can offer the following advantages for both kits and dams: specific starter diets could better cover kit nutritional requirements (De Blas *et al.,* 1999a; Gidenne and Fortun-Lamothe, 2002; Gutiérrez *et al.,* 2002) and shorter lactation periods could reduce the doe energy output for milk production and, consequently, body energy deficit (Parigi Bini and Xiccato, 1998).

Recent studies have compared rabbit does mated at 21 days and weaned at 42 days of lactation (traditional management) with those mated at 14 days and weaned at 35 days (semi-intensive management). The results indicate a lower number of kits at weaning (by 36%), a prolonged parturition interval and poorer feed efficiency with traditional management (Guillén *et al.,* 2008). In line with this, Martínez-Vallespin *et al.* (2011) reported that rabbit does under longer exposure to enviromental challenge show an adaptation process after 3 cycles. In fact it was described that delayed weaning implies more physiological effort for animals, which does not negatively affect productive and reproductive traits during the first cycles. However, this technique clearly implies reduced litter weight, less milk yields and increased live weight during in the following cycles, but preserves body condition (**Figure 49**). In the same vein, Savietto *et al.* (2011) reported that previous exposure and training seem to determine the response of animals when confronted with an environmental challenge, such as less nutrient availability than that required.

Many studies have reviewed the main factors affecting the reproductive traits of rabbit does. In the last 15 years, the profitability of rabbit farms has increased, mainly due to improvements in management and genetic selection, but several problems related to animal welfare have also emerged (Castellini *et al.,* 2010). Nowadays in reproductive rhythms, lactation and gestation clearly overlap. The resulting energy and hormone antagonism lowers the fertility rates and shortens the lifespan of does. Strategies to optimize these parameters are required to meet these objectives. Hence it is necessary to choose reproductive management that is more adapted to does' physiology (Castellini *et al.,* 2010).

Several studies have demonstrated the effects of different reproductive rhythms on rabbit does' reproductive performance and have shown how intensive management rhythm leads to decreased reproductive parameters, such as litter viability (Rebollara *et al.,* 2009). Likewise in primiparous rabbit does submitted to early weaning, Sakr *et al.* (2010) reported that despite early weaning at 25 dpp apparently improving the body energy stores of primiparous does if compared to a semi-intensive rhythm, this fact is not well reflected in ovarian

performance, which was significantly poorer than in the semi-intensive rhythm. García-García *et al.* (2009) obtained similar results with rabbit does, under transient litter separation during their reproductive life, exhibiting serum estradiol and testosterone concentrations and oocyte quality, which were influenced by intensive rhythm, leading to decreased reproductive parameters.

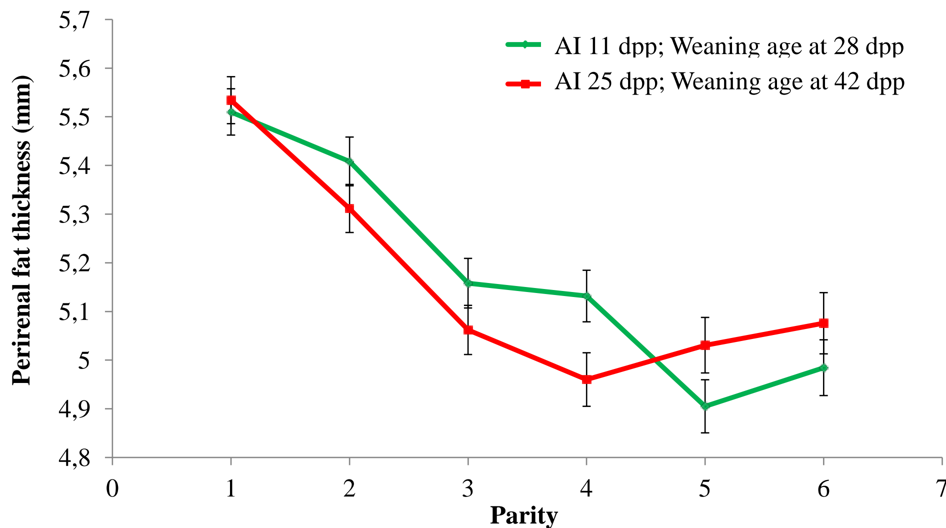


Figure 49. Evolution of body weight of rabbit does with short lactation (artificially insemination (AI) at 11 days post-partum (dpp) and weaning age at 28 dpp) and prolonged lactation (AI at 25 dpp and weaning age at 42 dpp) (Martínez-Vallespin *et al.,* 2011).

3.1.2. Effect of reproductive rhythm on the female immune system

Stress has been documented to have a negative impact on the immune system of both humans and animals (Kort, 1994; Agarwal and Marshall, 1998; Sapolsky *et al.,* 2000, Wu *et al.,* 2000; Reiche *et al.,* 2004; Sapolsky *et al.,* 2004; Glaser and Kiecolt-Glaser, 2005; Saul *et al.,* 2005). In cattle, the research measuring stress-related immune function focused on a number of husbandry management practices, including castration (Fisher *et al.,* 1997; Earley and Crowe, 2002; Ting *et al.,* 2003; Pang *et al.,* 2009; Marti *et al.,* 2010; Ting *et al.,* 2010; Pang *et al.,* 2011), housing (Fisher *et al.,* 1997; Hickey *et al.,* 2003b; Gupta *et al.,* 2007a), transport (Early *et al.,* 2006; Earley and O'Riordan, 2006; Buckham *et al.,* 2007; Gupta *et al.,* 2007b; Arthington *et al.,* 2008; Buckham *et al.,* 2008; Earley and Murray, 2010) and weaning (Hickey *et al.,* 2003a; Arthington *et al.,* 2005; Blanco *et al.,* 2009; Carroll *et al.,* 2009; Lynch *et al.,* 2010a, b; Enríquez *et al.,* 2011; Kim *et al.,* 2011; O' Loughlin *et al.,* 2011).

The effect of weaning on immunological parameters in other species (mice: Kato *et al.,* 2007; Probert *et al.,* 2007; swine: Brown *et al.,* 2006) and on physiology traits (Burke *et al.,* 2009) has also been described.

Hickey *et al.* (2003a) found that alterations in the immune function and hormonal mediators of stress were still present at 7 days after abrupt weaning, whereby cows were suddenly removed from calves. O' Loughlin *et al.* (2011) reported that weaning stress led to a number of modifications to the immune system; an earlier and more marked increase in the neutrophils number and a drop in the lymphocytes number in calves penned further away if

compared with calves penned near their dams post-weaning. These modifications suggest that the former may be more sensitive to weaning stress. No studies into the effect of weaning on rabbit immunological parameters have been found.

3.2. **Effect of environmental temperature**

The environment is made up of a number of components, such as temperature, humidity, air flow, dust, ammonia and gases, which can all affect the immune system.

Rabbits exhibit very low tolerance to the dust inevitably present in crumbly granulated feed, pollen and dust in the air from dry sweeping or being located near a dirt road. Dust has been reported to cause common coryza through the reflex action of the pituitary membrane, while ammonia and gases forming from decomposing, urine-soaked straw litters may quickly break down the pituitary membrane and gain direct access to lungs (Lebas *et al.,* 1997).

Temperature, humidity and air flow are three very closely linked environmental factors that are instrumental in triggering respiratory ailments. Air flow should not exceed 0.30 m/second, unless humidity is more than 75%. Ventilation errors in closed buildings are the chief cause of chronic pneumonia; in fact rabbits seem to be very sensitive to draughts (Lebas *et al.,* 1997). At lower temperatures, air must be correspondingly drier and move more slowly, but can also have negative effects on rabbits. However in the rabbit production context, heat stress is the main environmental factor on rabbit farms. The effect of heat stress is aggravated when heat stress is accompanied by high ambient humidity (Habeeb *et al.,* 1993; Marai *et al.,* 1996 and 2001).

The Earth's surface temperature has increased 0.8ºC over the last century. Consequently, future climate scenarios suggest that higher global mean temperatures could result in marked changes in the frequency of extreme temperatures (Crescio *et al.,* 2010).

The Mediterranean basin is considered a hotspot of biological diversity with a long history of modifications by human activities in natural ecosystems, and is one of the regions that is expected to face extensive climate changes (Maiorano *et al.,* 2011). The continuous rise in temperatures clearly affects the Mediterranean region, with extended summers (Coma *et al.,* 2009).

In Spain, environmental warming, even above the average, has been described (Ballester *et al.,* 2006). In fact in a report of 2005, the most favourable forecast for the Iberian Peninsula suggested an increase of 0.4ºC per decade in winter and of 0.6ºC per decade in summer during this century (Moreno, 2005).

3.2.1. Effect of heat stress on the rabbit performances

Apart from genetics and management, temperature also plays an important role in animal welfare and health. The relationship between high temperature and mortality is well-established among people living in urban areas, but has been poorly investigated in livestock (Crescio *et al.,* 2010).

The negative effect of high ambient temperatures on reproductive processes is well-documented in different domestic species (Hahn and Gabler, 1971; El-Fouly *et al.,* 1977; Dollah *et al.,* 1990; Marai *et al.,* 2006; Tusell *et al.,* 2011). In particular, it has been reported

that hot environments have an obvious impact on animal reproductive traits as they can suppress ovarian cyclicity (Christenson, 1980; Sharma *et al.,* 2010), puberty (Kurowicka *et al.,* 2006), ovulation (Rozenboim *et al.,* 2007), spermatogenesis, fertility (Kunavongkrit *et al.,* 2005; Flamenbaum and Galon, 2010), oogenesis and embryogenesis (Putney *et al.,* 1989, Edwards and Hansen, 1997; Beere, 2004; Lawrence *et al.,* 2004), by reducing conception and pregnancy rates (Christenson, 1980; Putney *et al.,* 1989; Garcia-Ispierto *et al.,* 2007), and by also inducing excessive secretion of ovarian hormones (Sirotkin, 2010). Moreover heat stress directly affects animal physiological activity by increasing blood, rectal and uterine temperatures (Kurowicka *et al.,* 2006), productive traits, and also parameters such as milk production in dairy cattle (Kadzere *et al.,* 2002; West, 2003; Finocchiaro *et al.,* 2005; Collier *et al.,* 2006; Silankove *et al.,* 2009; do Amaral *et al.,* 2009, 2011).

The most obvious limitation for rabbit production in an area with a hot climate, and in highly sensitive rabbits to heat stress (Castello, 1984; Simplicio *et al.,* 1988; Finzi *et al.,* 1994), is that heat produces a series of drastic changes in their biological functions which, in turn, impair production and reproduction (Marai *et al.,* 1999; 2002a, b; 2004). Such detrimental effects during hot climate season become evident and can limit rabbits' breeding season to the hemisphere (Marai *et al.,* 1996).

In Europe, seasons are usually analyzed in terms of the combined effects of light and temperature. However, reproduction problems sometimes appear at the end of summer, but have no direct relation to temperature (Lebas *et al.,* 1986). In tropical and subtropical areas, high ambient temperatures are the major constraint on animal productivity (Marai *et al.,* 1995). The effect of heat stress is aggravated when heat stress is accompanied by high ambient humidity (Habeeb *et al.,* 1993; Marai *et al.,* 1996 and 2001).

In this sense, negative effects of thermal stress on reproductive traits have been investigated (Hahn and Gabler, 1971; El-Fouly *et al.,* 1977; Dollah *et al.,* 1990; Marai *et al.,* 2006; Tusell *et al.,* 2011). In female rabbits, a reduced conception rate, embryonic development, litter size, litter weight and age at puberty and increased pre- and post-weaning mortality by exposure to heat stress, have been reported (Marai *et al.,* 2002a).

The bibliography shows that some affected parameters also relate with growing performance: feed and digestible energy intake, litter size and growth, rabbit doe weight (Prud'hon, 1976; Eberhart, 1980; Chiericato *et al.,* 1992; Paci *et al.,* 1993; Fernández-Carmona *et al.,* 1995; Amici *et al.,* 1998; Marai and Habeeb, 1998; Fernández-Carmona *et al.,* 2003; Guillén *et al.,* 2008). Moreover, thermal stress has been reported to affect the thermoregulatory function (Marai *et al.,* 2007), motorial activity (Finzi *et al.,* 1994), immunological values (Franci *et al.,* 1996) and metabolic parameters. These increase the levels of glucose, urea and lactate, and also GOT, GPT and CPK activities (Abdelatif and Modawi, 1994). Contrasting results have been recorded by several authors (Chiericato *et al.,* 1994; Marai *et al.,* 1994; Amici and Merendino, 1996; Amici *et al.,* 1998; Marai *et al.,* 2007), who observed an immediate, sudden drop in glucose and cholesterol following exposure to heat stress. Besides Fernandez-Carmona *et al.* (1995) reporting a depression in feed intake and feed efficiency (**Figure 50**), they mention disturbances in metabolism of water, protein, energy and mineral balances, enzymatic reactions, hormonal secretions and blood metabolites under heat stress conditions.

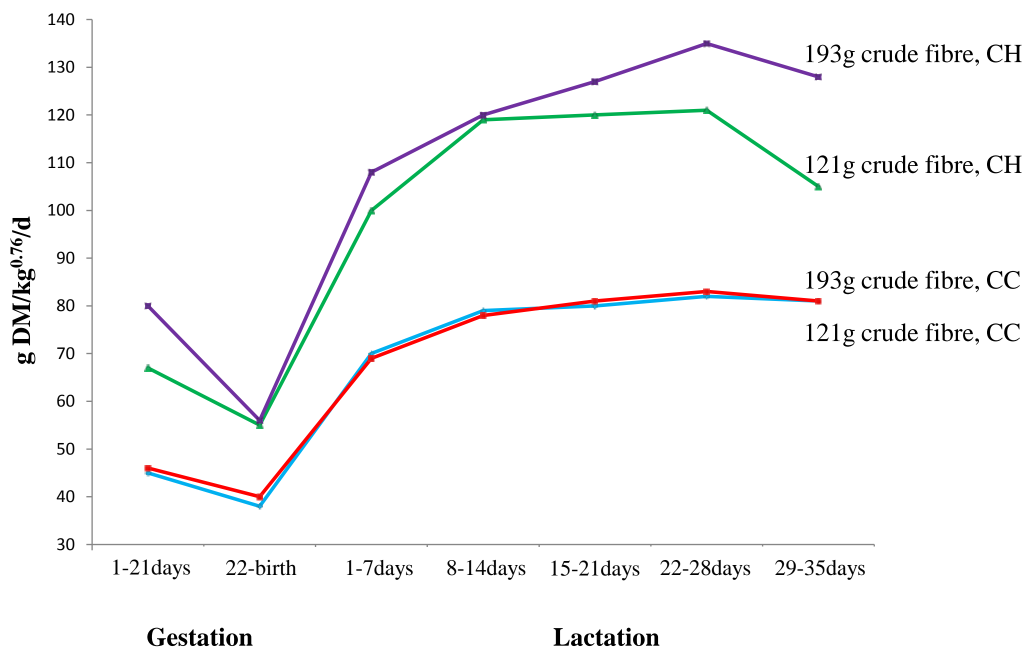


Figure 50. Effect of temperature on feed intake of breeding rabbit does. *DM*: Dry matter; *CC*: Climatic chamber (30ºC); *CH*: Conventional housing (Fernandez-Carmona *et al.,* 1995).

Regarding productive traits, several experiments carried out in summer in Spain or Egypt (Méndez *et al.,* 1986; Marai *et al.,* 2002b), or in climatic chambers (Fernández-Carmona *et al.,* 1995; Fernández-Carmona *et al.,* 2003) have shown that a hot climate reduces milk production and increases kit mortality. Guillén *et al.* (2008) described the same scenario for high temperatures, and reported that kit mortality at birth was high, and tended to be lower at first parturition as compared to the following two.

In several animal species, genetic differences in response to high temperatures have been observed (pigs: Zumbach *et al.,* 2008; cattle: Johnson, 1965; Ravagnolo and Misztal, 2002; Correa-Calderon *et al.,* 2004; Lacetera *et al.,* 2006; Aguilar *et al.,* 2009; Boonkum *et al.,* 2011; sheep: Pollot and Greff, 2004; and chickens: Khajavi *et al.,* 2003). These works have revealed that different individuals do not have the same responsive ability to face environmental heat challenges. The genetic selection for heat tolerance is possible, but continued selection for greater performance without considering heat tolerance leads to greater susceptibility to heat stress (West, 2003). In a study on heat stress in Mediterranean dairy sheep, which examined the relationship between milk production and heat stress, Finocchiaro *et al.* (2005) reported that milk yields were antagonistic with heat tolerance, and that selection only for increased milk production lowered heat tolerance.

When studying rabbit does facing an unexpected environmental challenge, Theilgaard *et al.* (2007) observed that a line selected by reproductive longevity criteria was less affected at its reproductive level (number of live births) than a line selected by litter size at weaning.

3.2.2. Effect of heat stress on the rabbit immune system

Very little information is available about immune response mechanisms at the cellular level by which rabbits overcome the functional and metabolic disorders produced by environmental stress. Nevertheless, numerous studies on the effects of environmental

temperatures over the upper critical temperature on immune cell functions in other species have been published. However, their results on the immune response are sometimes contradictory. In fact, some authors report improved immune systems (cattle: Soper *et al.,* 1978), some indicate no effects at all (human: Kappel *et al.,* 1991; cattle: Lacetera *et al.,* 2002) on lymphocyte functions following heat exposure, while others describe impairment (cattle: Elvinger *et al.,* 1991; Kamwanja *et al.,* 1994; Lacetera *et al.,* 2005; do Amaral *et al.,* 2011; poultry: Khajavi *et al.,* 2003). In particular, there are reports that heat stress affects immune system cells by directly lowering the number of both viable cells (Elvinger *et al.,* 1991; Kamwanja *et al.,* 1994) and receptors on immune cells' surfaces (Mehdi *et al.,* 1977; Kappel *et al.,* 1991). This implied reduced proliferative capacity of blood mononuclear cells (PBMC), especially in lymphocytes (Kamwanja *et al.,* 1994; Lacetera *et al.,* 2005, 2006; do Amaral *et al.,* 2010) and the neutrophil function (do Amaral *et al.,* 2011), which not only inhibits the differentiation of B lymphocytes into antibody-secreting cells (Franci *et al.,* 1996a), but lowers immunoglobulin and cytokines production (do Amaral *et al.,* 2010) and enhances the heat-shock proteins synthesis by lymphocytes (Rodenhiser *et al.,* 1985, Kamwanja *et al.,* 1994; Franci *et al.,* 1996b). In dairy cattle, Elvinger *et al.* (1992) reported that heat stress affects the immune function by reducing the migration of leukocytes to the mammary gland after a chemotactic challenge. This discrepancy among these results could be due to the diversity of the experimental approaches used. However, the results of *in vivo* studies (Lacetera *et al.,* 2005; do Amaral *et al.,* 2011) indicate that prolonged exposure to severe heat stress is responsible for diminished immune cell reactivity, which may contribute to some infectious agents occurring more frequently under extreme environmental conditions, exactly as under normal summer conditions (Cook *et al.,* 2002). Hence the expression of physiological functions, such as growth, immune competence and disease resistance, in any individual is an interaction involving genetics, nutrition and the environment (Thim *et al.,* 1997).

Heat-shock proteins (Hsps) (Lindquist, 1986; Benjamin *et al.,* 1990; Young, 1990), a group of proteins whose synthesis is enhanced by various forms of stress, form part of a general adaptive response that has evolved to protect prokaryotic and eukaryotic cells from stress (Li and Werb, 1982; Burel *et al.,* 1992; Heydari *et al.,* 1993). Hyperthermia influences the synthesis of these proteins, which have classified into five families according to their molecular weight (100, 90, 70, 60, and small Hsp; Kristensen *et al.,* 2004). Heat-shock proteins, particularly small Hsp such as Hsp27 and Hsp32, Hsp60, and Hsp70, play an important cytoprotective role during lung inflammation and injury (Wheeler and Wong, 2007) as they can activate the innate immune system by linking innate and adaptive immune responses (Anderson and Srivastava, 2000, Wallin *et al.,* 2002). Previous studies also indicate that Hsps play an immunoregulatory role in chronic inflammation (Hirayama, 2004; Van Eden *et al.,* 2007). Therefore, the analysis of anti-Hsp antibodies could prove to be an important tool to monitor an organism's ability to overcome stress and to maintain homeostasis, which affects animals' health and, consequently, farms' economic viability (Franci *et al.,* 1996).

In some species, several studies reveal the effects of thermal stress on the immune system, where circulating T lymphocyte counts lower (poultry: Guo and Gu, 1988; and sheep: Odongo *et al.,* 2006), as do natural killer cell counts and their cytolytic activity, cytokine secretion, lymphocyte proliferation and immunoglobulin concentrations (Shephard, 1998). Trout and Mashaly (1994) reported that helper T-cells ($CD4^+$) and cytotoxic T-cells ($CD8^+$)

decrease in the blood of heat-stressed birds. Accordingly, Khajavi *et al.* (2003) discovered that the percentage of CD4- and $CD8^+$ T-cells in the blood of chickens exposed to high temperatures significantly diminishes, and can impair the ability to produce antibodies and effective cell-mediated immunity.

Heat stress can negatively affect an animals' growth performance and immune competence to some bacterial or viral infections (Goligorsky, 2001; Odongo *et al.,* 2006). Heat stress has been reported to reduce: the weight of the thymus (Guo and Gu, 1988); in general, the weights of both primary and secondary lymphoid organs; the profiles of circulating leukocytes; T-cells in blood; and an antibody's response to red blood cells of sheep or against Newcastle disease (Donker and Beuving, 1989; Liew *et al.,* 2003). Hu *et al.* (2007) demonstrated that the chronic heat stress condition negatively affects both humoral and cellular responses against the foot and mouth disease virus in mice. However, there are very few detailed studies that address the effects of chronic heat stress on innate immune responses as the most immediate defence against viral infection. Only Jin *et al.* (2011) demonstrated that chronic heat stress can delay local immunity in the respiratory system in mice and rendered them susceptible to secondary infection of bacteria or viruses.

Some authors have also reported genetic variation in the ability to confront high temperatures and showed differences in the ability to minimize hyperthermia (Cartwright, 1955; Finch, 1986; Hammond, 1993) and cellular responses (Kamwanja *et al.,* 1994).

3.3. Effect of sporadic immunological challenge

3.3.1. Genetic variability for immune responses

In genetic selection programmes, the productive level of productive animals has considerable improved. However it is frequently observed in some species that selection by exclusive productive criteria has some associated effects, such as greater sensitivity to certain diseases (chickens: Burkhart *et al.,* 1983; pigs: Schinkel *et al.,* 1999; dairy cattle: Ravagnolo and Miztal, 2000; mice: Rauw *et al.,* 2002). This phenomenon is not new for industrial rabbits. Rabbit production has intensified in recent decades through improved genetic programmes, and to reproductive management and feeding systems, but changes in body condition and lifespan of females, and general health on farms, have also been associated (Rosell *et al.,* 2009; Pascual, 2010). In fact, rabbit health is considered a main handicap for current rabbit production under commercial conditions (Pascual, 2010).

In rabbit does, genetic selection programmes for reproductive traits have focused mainly on improving litter size at either partum or weaning (Pascual, 2010), but it remains unclear whether this selection criterion might affect the immune system response and adaptation capacity.

In other species, there is evidence that sensitivity to immune challenges may differ depending on genetic diversity (Rauw *et al.,* 1998; Salak-Johnson and McGlone, 2007; Siegel and Honaker, 2009). A study with cattle shows that Angus cattle exhibit a better Phytohaemagglutinin response than Braham × Angus crosses (Blecha *et al.,* 1984). Other studies indicate that IgG concentration differs between Angus and Hereford cattle (Muggli *et al.,* 1987), and that Angus cattle display a greater immune response than Simmental cattle (Engle *et al.,* 1999). Genetic differences in pig breeds have been reported in terms of

response to antigens or vaccines for red blood cells in sheep, *E. coli*, and other factors (Meeker *et al.,* 1987). Differences in NK cytotoxicity and lymphocyte proliferative responses have been found in two commercial lines of pigs (Reed and McGlone, 2000).

In line with this, a rabbit line founded by reproductive longevity criteria (Sánchez, 2006) revealed that the particular management of reserves by these animals makes them more robust and able to withstand environmental and productive challenges (Theilgaard *et al.,* 2007, 2009), which could explain their greater life expectancy on farms (Sánchez *et al.,* 2008). Works have also evidenced how usual long-term productive efforts expected of reproductive rabbit does could affect their health status. Thus, Martínez-Vallespín *et al.* (2011) reported greater physiological wear and higher culling rates for rabbit females subject to more demanding conditions (worse feed and delayed weaning age) **Figure 51**.

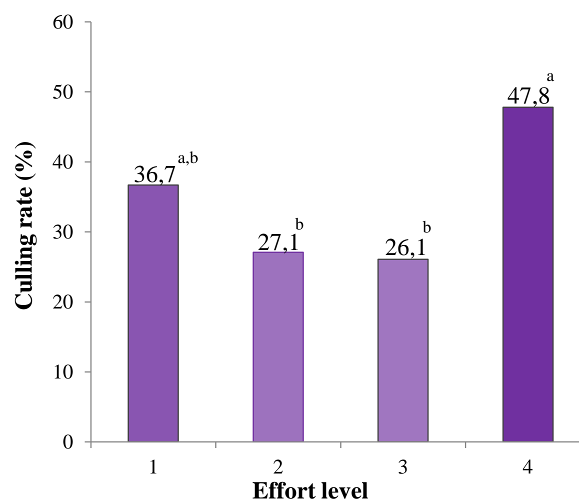


Figure 51. Culling rate for rabbit females at different reproductive and nutritional effort levels. *Effort level 1*: Standard feed and weaning age at 28 days post partum (dpp); *Effort level 2*: Low nutrient density feed and weaning age at 28 dpp; *Effort level 3*: Standard feed and weaning age at 42 dpp; *Effort level 4*: Low nutrient density feed and weaning age at 42 dpp. [a,b] The bars not sharing any superscript significantly differ ($P<0.05$) (Adapted from Martínez-Vallespín *et al.,* 2011).

3.3.2. Effect of lipopolysaccharide infusion on the rabbit immune system

A common way to simulate or induce immunological challenges is the infusion of lipopolysaccharide (LPS) from the bacterial wall of some pathogens, such as enteropathogenic *Escherichia coli.*

Bacterial LPS is a constituent of the outer leaflet of the outer membrane of Gram-negative bacteria (Nikaido and Nakae, 1979; Marca *et al.,* 2009), which has long since been acknowledged as an important determinant of both the virulence of these organisms and the symptomatology accompanying Gram-negative infection (Marshall and Ziegler, 1989). Bacterial LPS is able to induce a septic state and the release of several pro-inflammatory cytokines (Marca *et al.,* 2009). Secreted toxins are broadly known as "exotoxins", and the toxic materials of bacteria are termed "end toxins" (Wang and Quinn, 2010). Endotoxins are the key molecules in the induction of inflammation and the inflammational response in animals; furthermore, and pathophysiologically, the response is dose-dependent (Sandholm and Pyörälä, 1995; Burvenich *et al.,* 2007).

The LPS molecule can be divided into three parts: a lipid moiety, known as lipid A, core polysaccharides and O-antigen repeats (**Figure 52**). Lipid A represents the hydrophobic component of LPS, while core polysaccharides and O-antigen repeats are displayed on the surface of bacterial cells (Raetz and Whitfield, 2002; Raetz *et al.,* 2007). Lipid A is responsible for the toxic effects of infections with Gram-negative bacteria (Galanos *et al.,* 1985). In small doses in a localized tissue space, LPS signaling is advantageous to the host in orchestrating appropriate antimicrobial defense and bacterial clearance mechanisms. Conversely, the sudden release of large quantities of LPS into the bloodstream is clearly deleterious to the host as it triggers the release, in turn, of a dysregulated and potentially lethal array of inflammatory mediators and procoagulant factors in systemic circulation (Opal, 2010).

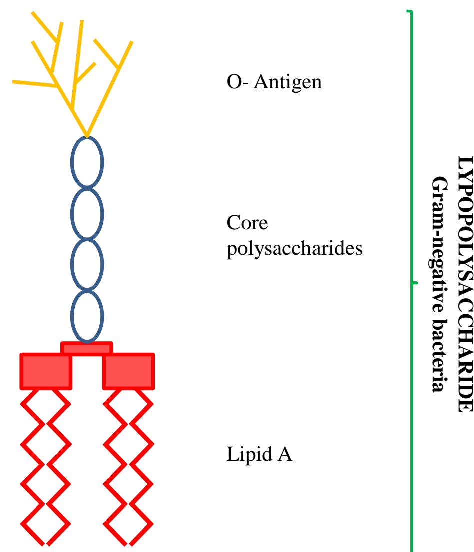


Figure 52. Structure of a bacterial lipopolysaccharide (LPS).

The detailed lipopolysaccharide structure differs from one bacterium to another, consistently with the recent discovery of additional enzymes and gene products that can modify the basic structure of lipopolysaccharide in some bacteria, especially pathogens. These modifications are not required for survival, but are tightly regulated in the cell and are closely related to the virulence of bacteria (Wang and Quinn, 2010).

Effects of Gram-negative bacterial LPS and dietary proteins on rectal temperature, serum hormones and haptoglobin have been evaluated in steers. Rectal temperature and serum cortisol, prolactin, haptoglobin and insulin increase, while glucose initially rise to then lower, and serum thyroxine and triiodothyronine drops for +LPS vs. -LPS steers (Waggoner *et al.,* 2009). Carroll *et al.* (2009) further characterized the acute-phase response following endotoxin exposure in bovines. Exposure to LPS from *Escherichia coli* increases: serum concentrations of cortisol, tumor necrosis factor-alpha (TNFα), interleukin 1-beta (IL-1β), IL-6, interferon-gamma (IFNγ), serum amyloid A respiration rate, rectal temperature. Rump skin temperature also increases following LPS administration. Recently, Yates *et al.* (2011) reported the effects of a bacterial LPS injection on white blood cell counts, hematological parameters and serum glucose, insulin, and cortisol concentrations in ewes. LPS has been seen to increase rectal temperature in a dose-dependent manner mostly between 2 and 24 h after inoculation. Total white blood cell concentrations are not affected by LPS, but LPS

increases neutrophil and monocyte fractions of white blood cells at 12 h and 24 h, and at 24 h after inoculation. Moreover, the lymphocyte fraction increases at 2 h, but decreases at 12 and 24 h after inoculation.

Bacterial LPS administration elicits a complex acute phase response (Krueger and Majde, 1990), which can be followed by changes in temperature (fever) and blood concentration of some physiological metabolites and acute phase proteins.

Leininger *et al.* (2000) demonstrated that an immunological challenge by lipopolysaccharide (LPS) from *Escherichia coli* in three genetic populations of certain high lean gain swine induces different changes to the swine immune system.

Many studies in rabbits have been carried out on immunological responses after a pathogenic challenge by bacterial LPS (Mashburn *et al.,* 1984; Mackiewicz *et al.,* 1988; Kimura *et al.,* 1994; Mo *et al.,* 1999; Shibata *et al.,* 2005; Sevillano and Reyna Sanchez, 2007; Huang *et al.,* 2008; Shao *et al.,* 2011). Yet very little is known about how pathogenic challenges affect the immune system, the physiology and use of resources available to animals of different genetic lines in rabbit does.

Different bacterial LPS challenges in rabbits have been studied (Mo *et al.,* 1999; Amador *et al.,* 2007; Stehr *et al.,* 2008; Marca *et al.,* 2009; Sitina *et al.,* 2011), but no studies indicate the relation connecting temperature, glucose level, heat shock protein and non-esterified fatty acids (NEFAs) after an immunological challenge.

3.3.2.1. Effect of lipopolysaccharide infusion on body temperature

Following a challenge with an infectious or inflammatory stimulus somewhere on the periphery of the body, a number of responses is generated within the central nervous system (CNS). These brain-mediated signs of illness entail changes in neuroendocrine activities, including: activation of the hypothalamic-pituitary-adrenal axis, anorexia and adipsia, changes in sleep patterns, decreased locomotor activity, libido, social contacts and exploration, symptoms which are collectively termed “sickness behavior”, and fever, that is, a regulated rise of body core temperature (Kluger, 1991; Blatteis and Sehic, 1997; Dantzer *et al.,* 1998; Zeisberger, 1999). A reduced febrile response is believed to contribute to, at least in part, increased mortality and prolonged recovery from infections (Kluger, 1986). Administration of bacterial LPS induces the appearance of the so-termed "cytokine cascade" in the circulation, more or less concomitantly with the febrile response (Roth and De Souza, 2001).

In rabbits (Kluger and Vaughn, 1978), as in other species, there are reports indicating an initial positive correlation between fever magnitude and survival, but this correlation reverses when fever is too high during acute challenges, and high, maintained temperatures spell risk of death. Fever induces overproduction of glutamate, hydroxyl radicals and prostaglandin E-2 in the rabbit hypothalamus. It has been shown how a systemic administration of LPS from *Escherichia coli* (2 mg/kg) induces increased levels of body temperature and hypothalamic levels of both glutamate and hydroxyl radicals, accompanied by increased plasma levels of TNF-alpha, IL-1beta, and IL-6 (Huang *et al.,* 2008). Pretel and Reyna (2007) reported how colonic temperature significantly increases in its maximum range at 80 and 180 min in rabbits administered with LPS from *Salmonella enteritidis*.

3.3.2.2. Effect of lipopolysaccharide infusion on energy metabolites in plasma

Adipose tissue plays an important role in energy homeostasis; however, very little is known about its metabolic activity during critical illness or sepsis (Wellhoener *et al.,* 2011).

Glucose dyshomeostasis features among the most injurious metabolic disorders during endotoxaemia, and profound hypoglycemia is commonly observed before death from endotoxin shock in humans and animals (Berk *et al.,* 1970; Holtzman *et al.,* 1974; Hinshaw, 1976; Filkins, 1978; Miller *et al.,* 1980; Lang *et al.,* 1985; Fischer *et al.,* 1986; Meszaros *et al.,* 1987; Lang and Dobrescu, 1991a).

Many studies record how plasma glucose content increases immediately after bacterial LPS inoculation (Waggoner *et al.,* 2009; Stengel *et al.,* 2010; Bernhard *et al.,* 2012). Extensive liver damage has been evidenced after a bacterial LPS challenge in rats and mice as these animals presented diffuse severe degeneration and total depletion of glycogen in hepatocytes (Ferrante *et al.,* 1984). This fact, concomitantly with absence of feed consumption, can lead to pronounced hypoglycaemia (Ferrante *et al.,* 1984; Fukuzumi *et al.,* 1996; Leininger *et al.,* 2000) and NEFA mobilization (Webel *et al.,* 1997; Leininger *et al.,* 2000; Kushibiki *et al.,* 2009), which are commonly observed after LPS challenges in other species. Macrophages have been implicated as they possibly contribute to hypoglycaemia since the highest glucose consumption *in vivo* is observed in macrophage-rich organs (Meszaros *et al.,* 1987; Lang and Dobrescu, 1991b), where bacterial LPS accumulates (Mathison and Ulevitch, 1979; Freudenberg *et al.,* 1982). In rabbits, a bacterial LPS stimulus increases plasma glucose content (Kiviranta *et al.,* 1995). In fact, increased glucose utilization to support immune system functions, and reduced liver fatty acid oxidation immediately after infection, have been extensively described (Blackburn, 1977; Grunfeld and Feingold, 1992).

In swine, Leininger *et al.* (2000) demonstrated that an immunological challenge by LPS from *Escherichia coli* not only induces anorexia, a significant increase in body temperature and circulating TNF-alpha, cortisol, and NEFA, but reduces circulating glucose, insulin and IGF-1. LPS-induced hypoglycemia differed significantly between genetic lines.

3.3.2.3. Effect of lipopolysaccharide infusion on acute phase proteins in blood

Concomitantly with the multiple changes associated with infections and aggressions, including local inflammation, fever and leukocytosis, a group of non structurally related proteins present in blood and other biological fluids, collectively named Acute Phase Proteins (APPs), was quantitatively and qualitatively modified (Ceciliani *et al.,* 2012). The acute phase response in hepatocytes is stimulated and mediated by the release of cytokines (including IL-1 and IL-6), which are produced by monocytes and macrophages where the inflammation or infection site is located. Therefore APPs are highly sensitive, but non specific, biomarkers of inflammation habitually, are used to make diagnoses and prognoses and to monitor therapies, but can be used indirectly to assess health status (Gruys *et al.,* 2006; Eckersall and Bell, 2010).

It has been reported that this systemic response to disease leads to an increased liver production of APPs (Jain *et al.,* 2011) at an early stage, as observed in previous works with rabbits (Murray and Connel, 1960; Mackiewickz *et al.,* 1988; Baker and Long, 1990; Petersen *et al.,* 2004; Georgieva *et al.,* 2009). In general, the APP response is an innate reaction to tissue lesions and rapidly follows (6-12 h) after the onset of any disease by

compromising tissue homeostasis induced by a range of causes, including infection, inflammation, cancer and trauma (Chase *et al.,* 2012). This response can also be induced by injecting microbial molecules (peptidoglycan, lipopolysaccharide) and pro-inflammatory cytokines (Baumann and Gauldie, 1994; Gabay and Kushner, 1999; Moshage, 1997). The APP response involves substantial changes in the serum concentrations of numerous proteins, mainly resulting from changes in their hepatic synthesis rates, although other organs and tissues also show local APP responses (Skovgaard *et al.,* 2009).

Among APPs, Haptoglobin (Hp) is a hemoglobin (Hb) binding protein whose major function is to prevent heme-iron-mediated oxidation. The polymorphic nature of the Hp gene results in varying levels of antioxidant functions associated with protein products (Goldenstein *et al.,* 2012). In fact it has been reported to serve as an immunomodulator and to possess anti-inflammatory and antioxidant functions (Abdullah *et al.,* 2012), which restrict the free iron need for bacterial growth (Eaton *et al.,* 1982). LPS alters the expression of a variety of genes, including transcription factors, cytokines, chemokines, receptors and cationic antimicrobial peptides (Rosenberger *et al.,* 2000) by showing an increase in APPs, especially haptoglobin (Turner *et al.,* 2002). The serum concentrations of two APPs, haptoglobin and serum amyloid-A, were monitored in reindeer following a challenge with endotoxin. The endotoxin challenge significantly increased the mean haptoglobin concentration at 8 h, which remained high up to 48 h (Orro *et al.,* 2004). Several studies in pigs have shown how the plasma haptoglobin response to LPS differs depending on genetic diversity (Leininger *et al.,* 2000).

The classical acute-phase reactant C-reactive protein (CRP) is an APP composed of five identical globular subunits, arranged as a cyclic pentamer (Ji *et al.,* 2006). Its basic features include control of inflammation, stimulation of the clearance of damaged cell and tissue components, and initiation of repair functions. CRP shows a calcium-dependent affinity to phosphate monoesters, such as phosphatidylcholine, but several other ligands of CRP have been characterized, including damaged cell membranes, small ribonucleoprotein particles, apoptotic cells and fibronectin (Biró *et al.,* 2007). In particular, CRP recruits C1q to the surface of damaged cells, thereby initiating complement activation (Mihlan *et al.,* 2011).

4. GENETIC SELECTION, BODY CONDITION AND HEALTH IN REPRODUCTIVE RABBIT DOES

As previously mentioned, rabbit health is considered a main handicaps for current rabbit production under commercial conditions. The appearance of a new challenge, such as epizootic rabbit enteropathy (ERE), usually requires more scientific effort to reduce its economical impact. In recent years, many studies have focused on acquiring more knowledge on the influence of nutrition on intestinal health in growing rabbits (Gidenne and Garcia, 2006; Carabaño *et al.,* 2008, 2009). Such efforts should not steer us away from studying current breeding systems as reproductive rabbit does can unbalance nutrient partitioning, and have an impact on global farm health and welfare, possibly involving the incidence of specific illnesses. In the 20 years, more or less traditional production systems have moved to other more intensive ones which employ modern prolific lines. Such action has led to adjusting the nutritional requirements that animals need in line with new demands (Maertens, 1992). In weaned rabbits, nutritional requirements have changed (between +0.45 and 1.23 g/day per generation of selection) as a result of selection by growth rates. However, reproductive does have suffered the effects of these improvements and new production systems to a greater extent.

Genetic selection programs in reproductive rabbits have focused mainly on improving litter size at either partum or weaning. These programs have effectively led to an increase of between 0.05 and 0.13 live-born kits per generation of selection (Rochambeau *et al.,* 1994; Gómez *et al.,* 1996). This selection criterion, along with the artificial insemination (AI) of does with semen from males selected per growth rate, has clearly increased the litter's milk demand. Moreover, intensification of reproductive rhythms leads to competition between mammary glands and fetuses, which is usually detrimental to fetal growth if needs are not met (Fortun and Lebas, 1994). Reproductive rabbit does' requirements may, therefore, have increased considerably in recent years, and could affect nutrient partitioning by compromising body condition, lifespan and general health on the farm. In line with this, some recent works suggest a possible effect of the doe health status on the potential risk of their kits having digestive troubles during the growing period (Quevedo *et al.,* 2003; Garcia *et al.,* 2005).

For this reason, and in the current productive context, Pascual *et al.* (2012) proposed that new breeding systems must be better defined in terms of animal welfare and the general health status on the farm. In this new suitable strategy, the feeding and genetic selection of reproductive does must be considered short-term (litter size, milk production, intervals between parturitions, etc.) and long-term (does' body condition, life expectancy and health status, etc.) productive criteria, while evaluating possible effects on subsequent litter development (transition at weaning, gastrointestinal health, etc.).

Both reproduction (litter size, milk yield, fertility, etc.) and survival (health, welfare, lifespan, etc) are energetically expensive. In mammals, body reserves are especially involved in successful reproduction and in maintaining the soma and, therefore, survival (Theilgaard, 2006). Thus, rabbit females' body condition might prove an important factor when addressing the association among reproduction, health and survival, and may consequently play a central role in defining their adequate genetic selection programmes (Pascual *et al.,* 2012).

4.1. Nutrient partitioning

The different metabolic functions of a rabbit female must be covered from available resources, and the processes by which available nutrients are channelled, in varying proportions, to these functions are known as nutrient partitioning (Friggens and Newbold, 2007). Nutrient partitioning changes, as far as the physiological stage is concerned, links changes in the endocrine profile (Bauman, 2000), which controls body fatness throughout the reproductive cycle (Vernon *et al.,* 2001), and in animal age, which are relative priorities of females for the different life function changes occurring throughout their lifetime (Martin and Sauvant, 2010). Thus, it is well-accepted that resources allocation between functions, and consequently body condition, must be genetically driven.

If there is a genetic component driving temporal changes of body reserves, then the evaluation of sporadic negative energy balances in female rabbits must be done as animal malfunctioning and as a possible natural adaptation to maximize her chances of evolutionary success (Friggens and Newbold, 2007). From an evolutionary viewpoint, rabbit selection has been addressed to maximize the number of viable litters produced during a lifetime. To achieve this goal, the optimal trade-off must be defined between number of pregnancies and postnatal investment in litter viability (Friggens, 2003); i.e., the optimal trade-off between maternal investment addressing the current and the future litter.

Based on these considerations, Pascual *et al*. (2012) suggests that the traditional view of body reserves mobilisation in reproductive rabbit does as a response of feed intake must be moved to an animal viewpoint, where feed intake must be considered more an “output” resulting from resources allocation in females to ensure the viability of current and future litters.

4.2. Body condition and reproduction

As previously mentioned, there is much evidence for a genetic component controlling size and mobilization of body fatness, and priority to safeguarding animal body reserves (Theilgaard, 2006). Not all mobilizations occur in response to environmental constraints (Friggens, 2003). In rabbits, some indications also suggest that the level of body reserves in a given physiological stage is highly defended. The vast majority of mammals have evolved astrategy to accumulate fat reserves during pregnancy (Gosling *et al.,* 1984; Chilliard, 1986, 1987; Ofteday, 2000). From the end of gestation, however, a drive appears to reduce body fatness in favor of the current litter despite the nutritional resources available. Therefore, the female body condition changes during the reproductive cycle and throughout her reproductive life, according to her genetically determined level. Problems appear when animals are forced to deviate from this adequate level due to increased susceptibility to disease, other stress factors and eventual failure (Friggens, 2003).

Different authors suggest that the relative risk of culling is higher during gestation and that it peaks around kindling (Theilgaard *et al.,* 2006; Rosell and de la Fuente, 2009; **Figure 53**). Negative effects for health may emerge in animals with excessively low or excessively high fat reserves during gestation. Particularly excessively high fat reserves at parturition may be at greater risk of metabolic diseases and lower feed intake during early lactation (pigs: Revell *et al.,* 1994; Brandt *et al.,* 1999; cows: Broster and Broster, 1998; rabbits: Pascual *et al.,* 1999).

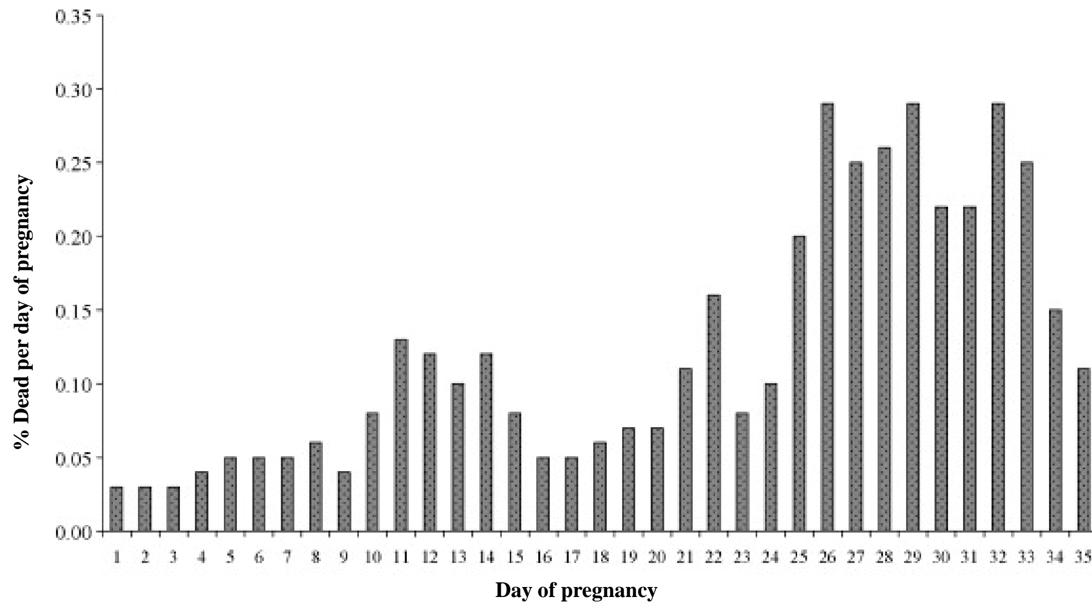


Figure 53. Percentage of dead females per day of pregnancy (mortality risk per day × 100 females). Data from 18 commercial farms with 366,162 pregnant females at risk and 16,339 removed dead (Rosell and de la Fuente, 2009).

On the other hand, Theilgaard *et al.* (2006) observed a higher relative risk of culling for reproductive does with a lower fatness level, suggesting that there is an optimum level of fat reserves where reproduction has a lower cost, and perhaps the animal deviates from this optimum level, which can have negative consequences for reproduction (**Figure 54**).

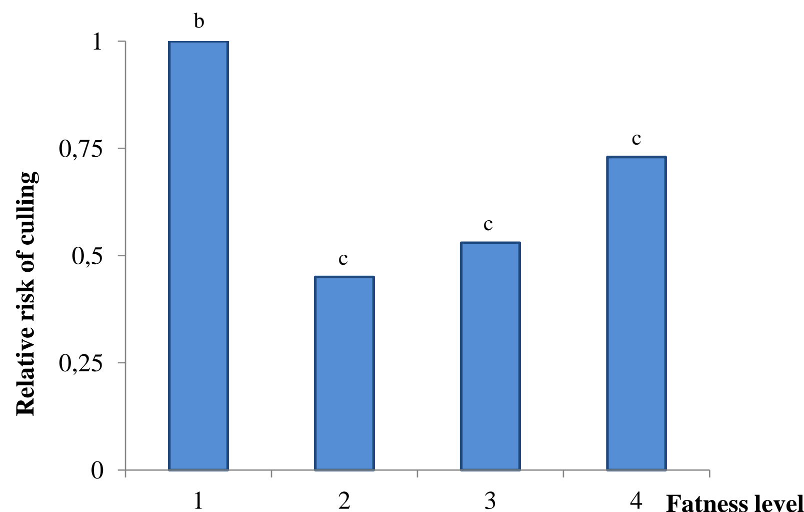


Figure 54. Relative risk of culling for reproductive rabbit does as regards level of fatness. [b,c] Bars not sharing any superscript are significantly different (Theilgaard *et al.,* 2006).

4.3. Genetic selection and resources allocation

4.3.1. Selection for litter size

From the above deductions for body condition and reproduction relationship, selection for improved litter size (reproduction), frequently used in rabbits (García and Baselga, 2002a, 2002b; Tudela *et al.,* 2003), can be expected to change rabbit females' ability to obtain resources. Conversely, negative consequences for body condition and survival are expected.

Different authors (García-Ximénez *et al.,* 1996; Quevedo *et al.* 2005, 2006) used freezing and transfer techniques to study the effect of selection for litter size upon weaning on rabbit does' performance and physiological and productive characteristics by the contemporary comparison of crossbred does with 12 generations of differential selection. They found that current females produce more live-born kits, and present higher feed intake and milk production during the first part of lactation as compared to older animals. These results could explain a possible change in animals' use of available resources as a result of selection. Hence, when selecting animals by litter size upon weaning, we select both prolificacy (more kits are born) and maternal aptitude criteria (survival of kits). Survival during lactation is determined mainly by what happens on day 1 post-parturition, and it clearly relates with kits' energy ingestion during this period, which is why increased milk production as a result of does' greater ingestion is favorable.

Mammals have evolved a breeding strategy whereby they fuel their reproduction from energy gained earlier, which is stored in body reserves. Body reserves are subsequently used to sustain the reproduction cycle, usually at the time of greatest energy demand; e.g., to ensure fetal growth at late pregnancy or nursing in the form of milk for litters at early lactation. Selection experiments evidence a relationship between reproduction and body reserves because selection for prolificacy increases litter' energy demand; selection for litter size, therefore, increases body fatness (e.g., pigs: Holl and Robinson, 2003; Estany *et al.,* 2002). In fact, Quevedo *et al.* (2005) demonstrated enhanced efficacy of use of energy feed for fetal growth as a result of selection by reproduction criteria since the product of pregnancy was clearly higher in selected does, despite not showing greater energy ingestion or greater mobilization of reserves. In addition, Quevedo *et al.* (2006) observed that when more animals were selected for litter size, they presented more perirenal fat thickness on day 10 of lactation, considered to be the maximum body condition day. Thus, the selection of animals by reproductive criteria might produce a response that correlates with animals' capacity to obtain resources (van Noordwijk and de Jong, 1986; Reznick *et al.,* 2002).

Similar results were recently obtained by Savietto *et al.* (2013b), where more selected rabbit females (plus 20 generations of selection for litter size upon weaning) under unlimited resources conditions brought about better daily feed intakes and milk yields during the first week of lactation, but lower milk yields during the last week of lactation. Friggens (2003) proposed that maternal investments in current litters peak at around parturition, which contributes to newborn litter viability (**Figure 55**).

Selection for litter size upon weaning may affect the relative priority for current litters through loss of shape (body weight) around kindling, which ensures the adequate development of larger, but less mature, litters. Increasing priority for current litter at the end of pregnancy implies more effort to ensure adequate fetal development, which may relate to

not only much less lactation effort at the end of lactation during the previous reproductive cycle, but also to the high mobilization observed at the end of pregnancy in this species (Savietto *et al.,* 2013b). In addition, loss of priority shape allows greater maternal investment after parturition (higher milk yields) as the kit survival index is determined mainly by what happens during early life, and clearly relates to kits' energy intake on these days (Quevedo *et al.,* 2006). Afterward, priority for current litters lowers as producing milk in excess of that required for litters, which progressively depend less on mothers' milk, does not improve the litters' chances of survival.

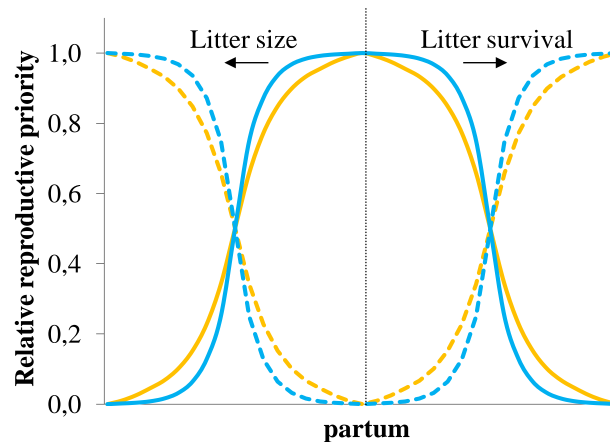


Figure 55. Proposal of Savietto *et al.* (2013b) for the evolution of relative reproductive priority for current [less (——) and more (——) selected for litter size] and future litters [less (- - - -) and more (- - - -) selected for litter size] in rabbit females from conception to weaning. Priority according to current litters is assumed to be the maternal investment rate, scaled from 0 to 1, as proposed by Friggens (2003).

Therefore, when prolific animals are selected for reproduction, those animal characteristics ensure that their selection success seem to be enhanced, which could be called "the number"; e.g., prolificacy and the ability to manage resources more appropriately when they are not limited in order to ensure the viability of larger litters, but with no negative effects on future ones.

However in a nutritional-restricted environment, Savietto *et al.* (2013b) observed how more animals selected for litter size upon weaning took longer to adjust their feed intake to compensate low dietary DE content, which also led to lower milk yields. Conversely, the main female body traits controlled were less affected by feeding restrictions in more selected animals. These results suggest that selection for litter size increases animals' sensitivity to this type of environmental challenge, leading to changes in nutrient partitioning and reduced performance in this restriction situation (Pascual *et al.,* 2012).

As proposed earlier, the fitness characteristic enhanced by this selection criterion is "the number". When available resources are presently limited, current litters' success can be questioned. Thus, animals probably decide to reduce the priority for current litters (by relatively addressing lower amounts of resources to those traits directly related to current litter performance, such as energy intake and milk yield), while the next litter seems to be prioritized (by relatively increasing fuel for future litters, body reserves). Therefore, when

animals are selected for reproductive traits, such as litter size, and available resources are presently limited, they cannot ensure current litters' success as maternal investment in current litters lowers (consequently, animals are less robust or more sensitive to the environment) to concentrate efforts in ensuring future litters (Friggens, 2003) when perhaps both resources and the environment may improve.

4.3.2. Selection for longevity

Longevity reflects the animal's ability to not be culled or to die. The main culling reasons in animal production include diseases, low fertility and low production (Vollema, 1998). Recent decades have witnessed a considerable rise in livestock production per animal. This increase is due largely to successful selection for productive traits (e.g., milk yield, growth, litter size) in combination with improved dietary formulation and management. Focusing almost exclusively on production traits has negative side effects, such as lower fertility, higher frequency of metabolic diseases for animals and less offspring viability. However, there are frequent reports of positive relationships between productive traits and longevity (Lynch and Walsh, 1998). Thus, when to expect positive or negative relationships among production, reproduction and survival still remains unclear.

In rabbit production, the main traits of interest are growth rate, litter size and fertility. Selection for reproduction performance has not yet been reported to have negative effects on longevity. For example, there are some reports indicating that large litter sizes positively associate with longevity in meat type does (Garreau *et al.,* 2001; Sánchez *et al.,* 2006). Moreover, an experiment comparing a rabbit line selected for litter size over seven generations with a control line found no differences in longevity (Rinaldo and Bolet, 1988). The only work into rabbit production that estimates the genetic correlation between reproductive performance and survival found that this correlation was no different from zero (Sánchez *et al.,* 2006).

Recently, Theilgaard *et al.* (2007) evaluated genetic differences in reproductive performance and body condition traits during successive parities between a longevous productive (LP) line (constituted by the hyperselection of animals with an extremely large number of parities [at least 25] and average reproductive performance) and a line (V), selected for 31 generations for litter size upon weaning. Both lines were found to exhibit equal reproductive performance during the first three cycles. However, when animals were subjected to a non programmed restricted environment (change of nutritional management to feed restriction after weaning) on one of the farms as from the third reproductive cycle, litter size upon the birth of V line females was depleted from this time, while the prolificacy of the LP line ones was maintained at the expense of a reduction in their greater soma (**Figure 56**). Theilgaard *et al.* (2007) hypothesized that the higher body soma of adult LP females allowed them greater body buffer capacity, but diminished their environmental sensitivity.

Therefore, the hyperselection for reproductive longevity and average prolificacy could delay reproductive senescence as this newly founded line seems to show less environmental sensitivity. Nevertheless, no indication of a trade-off between reproduction and survival was found in these experiments in any case. When sufficient resources are available, females should be better able to meet both reproduction and maintenance demands without compromising either. Failure to provide enough energy for maintenance has a deteriorative effect on their physiological condition, and continued reproduction under such conditions is

likely to increase susceptibility to disease and other stress factors (Friggens, 2003). So, these animals might be less robust and show increased susceptibility to disease, other stress factors and eventual failure.

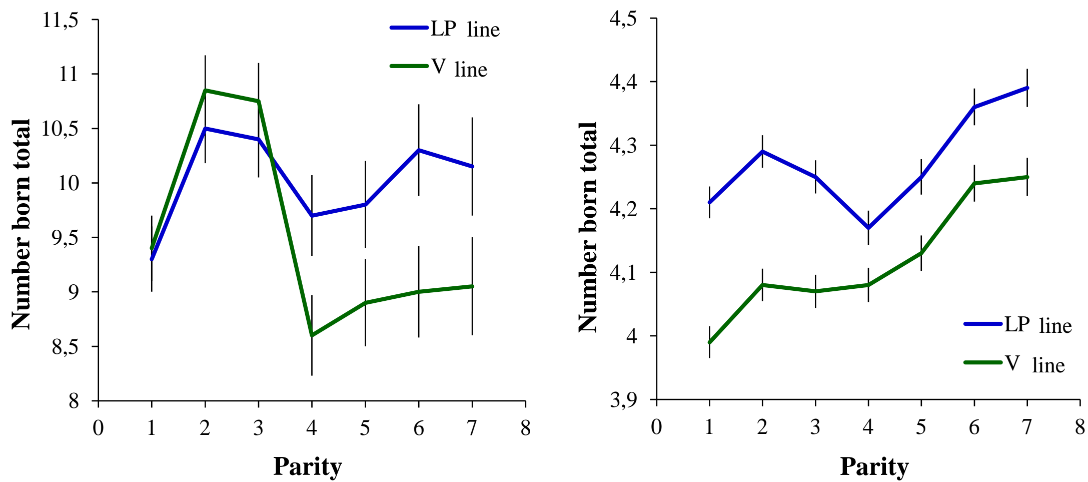


Figure 56. Effect of the genetic line on the evolution of the number of total born and acceptance rate. *LP*: Hyper-longevity productive; *V*: Reproductive (Adapted from Theilgaard *et al.,* 2007).

In rabbits, Theilgaard *et al.* (2007) observed that the LP line was heavier than the V line (4.27 *vs.* 4.12 kg; P<0.05) throughout six reproductive cycles, which is in agreement with experiments where selecting for reproductive longevity in mice (Nagai *et al.,*1995), and in late reproduction in flies (Partridge and Fowler, 1992), also increased body weight. These findings suggest that selection for longer life favors extended growth to produce a more durable adult soma.

To confirm these results, Theilgaard *et al.* (2009) compared the performance and body condition traits of LP and V does at different productive effort levels (previous AI at partum or weaning, and litter sizes of 5 or 9 pups) during their second lactation. Once again, LP does were significantly heavier than the V line ones, but they also showed a better body condition and lower mobilization at the beginning of lactation, thus confirming the greater soma of this type of animals to confront productive and environmental challenges. In fact, LP did not produce higher milk yields than V does, even per kg of metabolic weight, especially under more lactation pressure (9 pups).

Theilgaard *et al.* (2009) suggested that the greater soma of the rabbit females selected for reproductive longevity seemed to allow them to better cope with the possible productive challenges that they might encounter during their productive life. They seemed to present greater plasticity, which enabled them to use their greater soma to overcome these demanding situations. This scenario was reflected by the more the productive effort, the smaller the differences observed in body weight (BW) between lines. Therefore, Pascual *et al.* (2012) proposed that the rabbit females selected for reproductive longevity were more "robust" in these situations (more milk for offspring), and their risk of early culling for low productivity consequently lowered.

4.3.3. Robustness

It seems that when resources availability is not limited, more selected animals can successfully cope with most of their major needs without too many negative consequences. In fact, it is not unusual to find positive relationships between productive traits and survival for reproductive stock when animals are reared under a controlled environment; i.e., cows (Short and Lawlor, 1992), sows (Serenius and Stalder, 2004) and rabbit does (Theilgaard *et al.,* 2006). However, this is when animals suffer under sporadic, but not infrequent, and even cyclical, suboptimal environments (i.e., resources limitation, heat stress, immunological challenge), which is when high-producing animals are more sensitive to these stressing conditions (Schinkel *et al.,* 1999; De Greef *et al.,* 2001; Yalcin *et al.,* 2001; Windig *et al.,* 2005).

Knap (2005) defined the robustness concept in farm animals as 'the ability to combine a high production potential with resilience to stressors, allowing for an unproblematic expression of a high production potential in a wide variety of environmental conditions'. Another component of the definition of a female's lifespan is her sensitivity to common diseases, which may appear on rabbit farms. Possible changes in resources allocation, this being the result of genetic selection or reproductive management, could drive females to situations in which body condition can be withdrawn from an adequate level by increasing animals' susceptibility to sporadic immunological challenges. For example, the greatest drop in body reserves takes place at the end of gestation, which is when more commercial does are eliminated (Rosell and de la Fuente, 2009). These results could reflect the importance of not steering too far away from an appropriate body status to ensure reproduction and to lower the risk of elimination.

HYPOTHESES AND OBJECTIVES

B. HYPOTHESES AND OBJECTIVES

Throughout the literature review, how some productive conditions of modern rabbit production (high reproductive rhythms, exposure to sporadic heat stress conditions and genetic selection criteria) may affect both body condition and health status of females has been described. Thus, reproductive rhythm can affect risk of culling in rabbit does. For example, habitual weaning protocols are delayed to reduce mortality during the fattening period, probably because of the protective role of milk (Fortun-Lamothe and Boullier, 2007; Gallois *et al.,* 2007), and because later weaned rabbits present a more mature immunity. However, these late weaning protocols imply prolonged lactation, which might affect rabbit does' body condition (Pascual *et al.,* 2006). It has also been observed how the usual long-term productive efforts expected of reproductive rabbit does can affect their health status. Thus, Martínez-Vallespín *et al.* (2011) reported greater physiological wear and higher culling rates for rabbit females subject to high demanding conditions (poorer feed and delayed weaning age).

One of the main aims of this thesis was to study how productive effort (traditional and late weaning) and body condition may affect the evolution of lymphocyte populations in breeding rabbits.

On the other hand, the relationship between high temperature and health in livestock has been poorly investigated (Crescio *et al.,* 2010). There is clear evidence that heat stress affects the immune system of different species as it lowers the number of viable cells (Elvinger *et al.,* 1991; Kamwanja *et al.,* 1994) and receptors on immune cells' surface (Mehdi *et al.,* 1977; Kappel *et al.,* 1991). This scenario implies reduced proliferative capacity of blood mononuclear cells (PBMC), especially in lymphocytes (Kamwanja *et al.,* 1994; Lacetera *et al.,* 2005, 2006; do Amaral *et al.,* 2010). Moreover, the neutrophil function (do Amaral *et al.,* 2011), not only inhibits B lymphocytes differentiation into antibody-secreting cells (Franci *et al.,* 1996a), but diminished immunoglobulin and cytokines production (do Amaral *et al.,* 2010) and enhances heat-shock proteins synthesis by lymphocytes (Rodenhiser *et al.,* 1985, Kamwanja *et al.,* 1994; Franci *et al.,* 1996b). This is especially important in a global warming scene, together with economic and energetic crises in a country like Spain, where higher temperatures are recorded with each passing year. Hence, the second aim consisted in studying how genetic selection type (for reproduction or robustness criteria) and the environment may affect the evolution of lymphocyte populations of breeding does under normal and heat stress conditions.

Throughout genetic selection programmes, the productive level of animals has considerably improved. In rabbit does, genetic selection programmes for reproductive traits have focused mainly on improving litter size at either partum or weaning (Pascual *et al.,* 2010), but it remains unclear whether this selection criterion affects the immune system's response and adaptation capacity. Accordingly, a rabbit line founded for reproductive longevity criteria (Sánchez, 2006) has shown that the particular management of reserves by these animals makes them more robust and capable of withstanding environmental and productive challenges (Theilgaard *et al.,* 2007, 2009), which could explain their greater life expectancy on farms (Sánchez *et al.,* 2008). There is very little information available on such robustness from an immunological viewpoint. In fact, the effect of an LPS challenge in different species has been described and there is evidence that sensitivity to immune challenges may differ depending on genetic diversity (Rauw *et al.,* 1998; Salak-Johnson and McGlone, 2007;

Siegel and Honaker, 2009). It has also been reported that LPS administration elicits a complex acute phase response (Krueger and Majde, 1990), which can be followed, among others, by fever and changes in the blood concentration of some physiological metabolites and acute phase proteins. Different LPS challenges in rabbits have been performed (Mo *et al.,* 1999; Amador *et al.,* 2007; Stehr *et al.,* 2008; Marca *et al.,* 2009; Sitina *et al.,* 2011), but no studies report the relation linking temperature, glucose level, heat shock protein and NEFAs after an immunological challenge in different genetic lines of rabbit does. Thus, the third aim was to study how the genetic selection type and production levels may affect animals' immune response against a specific antigen such as LPS.

In the last three decades, rabbit meat production has shifted from traditional production systems to other more intensive ones relating to relevant advances in reproductive management, feeding systems and genetic selection, of which genetic selection has allowed increased production levels. Thus, intensification of production systems and genetic selection are strongly related. In parallel, the incidence of metabolic diseases and pathologies has also grown in general, thus compromising body condition, lifespan and general health on farms.

Therefore, the overall **thesis hypothesis** proposes that common challenges on modern rabbit production (reproductive effort, sporadic heat stress conditions or genetic selection for reproductive traits) could affect resources allocation in reproductive rabbit does (partitioning of available resources to vital functions). Addressing more resources to confront these challenges can reduce females' ability to respond to other demands such as coping with diseases. Thus, body condition may play a mediating role. Thus for animals at the same production and genetic level, this entails the following: the better the body condition, the better the immune response (**Figure 57**).

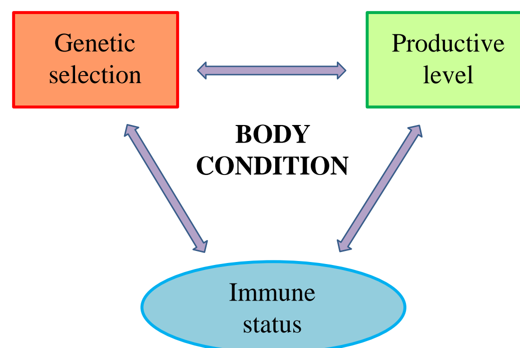


Figure 57. Relationship among genetic selection, productive level and immune status.

The objectives of this thesis were:

1- To know the effect of weaning age (28 vs. 42 dpp) on peripheral blood lymphocyte populations of multiparous rabbit does and their litters.

2- To characterize blood lymphocytes and their evolution from first to second parturition of rabbit does differing in animal type (by comparing two distant generations of a line selected for litter size at weaning and a line founded with reproductive longevity criteria) and to study animals' response to heat stress in terms of animal type.

3- To evaluate the effect of selection of rabbit does for either longevity and litter size or litter size at weaning on their immune responses to lipopolysaccharide.

MATERIALS AND METHODS

C. MATERIALS AND METHODS

1. STUDY DESIGN I

1.1. Animals

The study sample included 22 adult rabbit females (*Oryctolagus cuniculus*) crossbred from two maternal lines selected by litter size (A and V lines, Universidad Politécnica de Valencia, Spain), aged between 11 and 15 months. Animals were housed in flat-deck cages of 700 mm × 500 mm × 320mm, with a light cycle of 16 light hours and 8 dark hours under controlled environmental conditions.

Since their first parturition, at 5-months old, females were randomly allocated and maintained under one of the two possible reproductive management rhythms: insemination at 11 dpp and weaning at 28 dpp (11 animals, named 28D) or insemination at 25 dpp and weaning at 42 dpp (11 animals, named 42D).

After their fifth parturition, peripheral blood samples were taken at different times. For the 28D rabbit does, blood samples were taken at 16 dpp (around the maximum daily milk yield), 28 dpp (weaning), 35 dpp (during recovery of energy reserves) and 42 dpp (sixth parturition). With the 42D rabbit does, blood samples were taken at 16 dpp, 28 dpp, 42 dpp (weaning) and 49 dpp (during recovery of energy reserves). In both groups, blood samples from two young rabbits of each female were also taken at weaning.

All the blood samples were drawn from the median artery of the ear using vacuum tubes with EDTA. Diurnal variations in haematological parameters were minimised by collecting blood at approximately the same time (9:00–11:00 h).

The Committee of Ethics and Animal Welfare of the Universidad Politécnica de Valencia approved this study. All the animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005; BOE = Official Spanish State Gazette).

1.2. Haematological studies and flow cytometry analysis

Blood samples were processed 1 hour after sampling. Before performing the flow cytometry studies, a white blood cells (WBC) count and the percentage of lymphocytes were determined using a haematology analyzer (MEK-6410, Nihon Kohden, Japan).

After mixing by inverting the tube, 50 µL of whole blood were pipetted into flow cytometry tubes and primary monoclonal antibodies (**Table 7**) were added, following the manufacturer's recommendations, and incubated for 15 min at room temperature in the dark. WBC were isolated by lysing erythrocytes by adding 1mL of ammonium chloride lysing solution (8.02 g NH_4Cl, 0.84 g $NaHCO_3$ and 0.37 g EDTA per litre of Millipore water) at 4 ◦C. After a 5 min incubation in the dark, samples were centrifuged at 400×g for 5 min at room temperature, the supernatant carefully eliminated and the pellet washed with 1 mL of phosphate-buffered saline (PBS). After another wash, secondary antibodies (Rat anti-mouse IgG2a+b Phycoerythrinn [VMRD, Inc.-exalpha] and Goat anti-mouse IgM: R-Phycoerythrin-human adsorbed-[AbD Serotec]) were added. These were incubated for 20 min at room temperatura in the dark. Finally, 1 mL of PBS was added before running the

flow cytometer. The resulting WBC suspensions were analysed in a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA). Specific data acquisition protocols for rabbit WBC were designed using the CXP software (Beckman Coulter, Brea, CA). The common leukocyte antigen CD14 and CD45 expression was used for the "lymphogate" setup as previously described (Jeklova *et al.,* 2007a). Gates of each leukocyte type were adjusted with isotype negative control. All the samples were processed in duplicate.

The total lymphocyte count was calculated as the product of the WBC count and the lymphocyte percentage, and of the lymphocyte subset counts and percentages, as described by Hulstaert *et al.* (1994).

Table 7. Monoclonal antibodies used in this study.

Monoclonal antibodies	Isotype	Specificity	Cell labeling	Clone	References	Company
Mouse anti-rabbit T lymphocytes:FITC[a]	IgG1	CD5	T cell	KEN-5	Kotani et al. (1993a)	Abd Serotec
Mouse anti-rabbit α-pan B IgM	IgM	IgM	B cell	MRB143A	Davis and Hamilton (2008)	VMRD Inc.
Mouse anti-rabbit CD4	IgG2a	CD4	T cell subset	KEN-4	Kotani et al. (1993a)	Abd Serotec
Mouse anti-rabbit α-CD8	IgG2a	CD8	T cell subset	ISC27A	Davis and Hamilton (2008)	VMRD Inc.
Mouse anti-rabbit CD25	IgG2b	CD25	Activated T cells	KEI-ALPHA1	Kotani et al. (1993b)	Abd Serotec
Mouse anti-human CD14:FITC	IgG2a	CD14	Monocytes and granulocytes	TÜK 4	Jacobsen et al. (1993)	Abd Serotec
Mouse anti-rabbit α-CD45	IgM	CD45	All leukocytes	ISC76A	Davis and Hamilton (2008)	VMRD Inc.

[a] Clon KEN-5 recognises rabbit T lymphocytes and immunoprecipitates. This antibody recognises rabbit CD5, but does not bind to rabbit CD5 transfectants. Known rabbit CD5 antibodies also show binding to most B lymphocytes, which are not labelled by this clone (information obtained from datasheet).

1.3. Ultrasound measurements

The perirenal fat thickness (PFT) of does was measured at parturition and weaning by ultrasound to evaluate body condition, as described by Pascual *et al.* (2000, 2004). Fur was removed from the thoracic and lumbar vertebrae areas by shearing to improve image retrieval. Animals were placed in an immobilizing box (150 mm × 370 mm × 150mm) during the ultrasound measurement and ultrasound gel was applied to the scanning area. The probe was always placed in the same position to obtain a repeatable transversal section of perirenal fat at 3 cm ahead of the 2nd lumbar intervertebral space. Images were obtained with an ultrasound unit (JustVision 200, Toshiba, Japan) equipped with an image analyzer software to determine distances.

1.4. Statistical analysis

To analyse the evolution of the lymphocyte populations in the blood of multiparous rabbit does, a mixed model (PROC MIXED; Statistical Analysis System, 2002) was used in accordance with a repeated measures design that takes into account the variation between animals and the covariation within them. Covariance structures were objectively compared using the most severe criteria (Schwarz Bayesian criterion), as suggested by Littell *et al.* (1998). The model (1) included weaning age (28 and 42 dpp), days from partum (16, 28, 35,

42 and 49 dpp), and their interaction as fixed effects. Random terms in the model included a permanent effect of each animal (p) and the error term (e).

$$y_{ijk} = weaning_i + day_j + weaning_i \times day_j + p_k + e_{ijk} \quad (1)$$

The model used to analyse the data of the lymphocytes populations in the blood of young rabbits at weaning was a split plot design (PROC GLM; Statistical Analysis System, 2002) that included weaning age (28 and 42 dpp) as a fixed effect. Random terms in the model (2) included a permanent effect of mother (p) and the error term (e).

$$y_{ij} = weaning_i + p_j + e_{ij} \quad (2)$$

Finally, to test the relationship between the lymphocyte populations of multiparous rabbit does and both the PFTs of females during lactation and the lymphocyte populations of young rabbits at weaning, Pearson's correlation coefficients (ρ) were obtained using PROC CORR of the Statistical Analysis System (2002).

2. STUDY DESIGN II

2.1. Animals

A total of 65 female rabbits of two different genetic lines (20 and 45 females from LP and V, respectively) were used, with the participation of females of two generations (16th and 36th) of the V line (23 and 22 females from V16 and V36, respectively). The V line was selected for litter size at weaning using the best linear unbiased prediction (BLUP) as the selection criterion in a single-trait repeatability animal model (Estany *et al.,* 1989; García and Baselga, 2002a). The parents of the V16 females were stored as frozen embryos to be then thawed and transferred to obtain live adults, which allowed the constitution of V16 population by reproduction, population that were contemporary to the current generation (V36). The LP line was founded under the longevity and reproductive criteria (selecting females from commercial farms with at least 25 litters and with a minimum average litter size of 7.5 kits born alive), as described by Sánchez *et al.* (2008). Then females were selected for litter size at weaning during six generations (the average value for prolificacy in the Spanish commercial rabbit population is approximately 9 kits born alive per litter at an average of 6 parities; Ramón and Rafel, 2002).

The Committee of Ethics and Animal Welfare of the Universidad Politécnica de Valencia approved this study. All the animals were handled according to the principles of animal care published by the Spanish Royal Decree 1201/2005 (BOE, 2005; BOE = the Official Spanish State Gazette).

2.2. Experimental procedure

From 63 days of age until the first parturition, all the female rabbits were housed in conventional housing (with a light-alternating cycle of 16 hours of light and 8 hours of darkness, under controlled environmental conditions: average daily minimum and maximum temperatures of 14ºC and 20ºC, respectively), using individual cages (700×500×320 mm) provided with a nest for litters from gestation day twenty-eight. After first parturition, the animals from the three animal types (LP, V16 and V36) were randomly distributed into two different experimental housing systems: CH, where 33 females (10, 11 and 12 from LP, V16 and V36, respectively) were maintained in conventional housing at the average daily minimum and maximum temperatures of 14ºC and 20ºC, respectively; CC, where 32 females (10, 12 and 10 from LP, V16 and V36, respectively) and their litters were housed in a climatic chamber and were maintained with a sinusoidal daily curve from 25ºC to 36ºC. Litter size was standardized to 9 and 10 kits at first and second parturition, respectively, in both environments.

The climatic chamber was equipped with a heating/cooling system which scheduled a sine function for the daily environmental temperature, with a minimum temperature of 25ºC early in the morning and a maximum one of 36ºC in the afternoon. This system ensured environmental stress as a temperature up to 28ºC for 65% of the day was in use (see the technical details in García-Diego *et al.,* 2011). In brief, the indoor microclimate was monitored by means of three probes located 30 cm above the animal cages. An additional one was located outside the farm. Each probe contained a 1-wire protocol integrated circuit (model DS2438, Maxim Integrated Products, Inc.) incorporating a temperature sensor. This integrated circuit was designed for on-chip measurements of battery temperatures and

voltages. Probes were calibrated prior to being installed, as described in a previous study (García-Diego and Zarzo, 2010). Data were saved at a frequency of one datum per minute.

Until the first parturition, all the females received a rearing diet *ad libitum* (9 MJ of digestible energy (DE) and 133 g of digestible protein (DPr) per kg, dry matter (DM)). From this time, females and their litters were fed the same diet as lactating rabbit does (11.5 MJ DE and 120 g DPr per kg DM), which was provided *ad libitum* until the end of the experiment (second parturition). Does were artificially inseminated (AI) on day 11 post-partum (dpp) and successive inseminations were carried out every 21 days, when necessary. Litters were standardized at birth to 9-10 kits and weaned on 28 dpp.

To evaluate the possible correlation between the energy balance and the immunological status of females, daily feed intake (DFI) in lactation week two and perirenal fat thickness (PFT) on 14 dpp were controlled by ultrasounds (Pascual *et al.,* 2000) given the recovery of body reserves in rabbits in the first part of lactation (Quevedo *et al.,* 2006). Thus, feed intake and body reserves in the second week of lactation were expected to be crucial for the female rabbits (Theilgaard *et al.,* 2006).

Blood samples were taken from females at the first parturition (at the start of the environmental challenge), on 4 dpp (after a short exposure to the environmental challenge), on 10 dpp (close AI and maximum body condition during lactation), and at the second parturition (end of the experiment). All the blood samples were drawn from the median artery of the ear using vacuum tubes with EDTA. Diurnal variations in haematological parameters were minimized by collecting blood at approximately the same time (9:00–11:00 h).

2.3. Haematological studies and flow cytometry analysis

A flow cytometry analysis was carried out as previously described. Blood samples were processed 1 hour after sampling. Before performing the flow cytometry studies, a white blood cells (WBC) count and the percentage of lymphocytes were determined using a haematology analyzer (MEK-6410, Nihon Kohden, Japan).

After mixing by inverting the tube, 50 μL of whole blood were pipetted into flow cytometry tubes and primary monoclonal antibodies (**Table 7**) were added, following the manufacturer's recommendations, and incubated for 15 min at room temperature in the dark. WBC were isolated by lysing erythrocytes by adding 1 mL of ammonium chloride lysing solution (8.02 g NH_4Cl, 0.84 g $NaHCO_3$ and 0.37 g EDTA per litre of Millipore water) at 4ºC. After a 5-min incubation in the dark, samples were centrifuged at 400×g for 5 min at room temperature, the supernatant was carefully eliminated and the pellet washed with 1 mL of phosphate-buffered saline (PBS). After another wash, secondary antibodies (Rat anti-mouse IgG2a+b Phycoerythrin [VMRD, Inc. α-exalpha] and Goat anti-mouse IgM: R-Phycoerythrin-human adsorbed-[AbD Serotec]) were added. These were incubated for 20 min at room temperature in the dark. Finally, 1 mL of PBS was added before running the flow cytometer. The resulting WBC suspensions were analysed in a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA). Specific data acquisition protocols for rabbit WBC were designed using the CXP software (Beckman Coulter, Brea, CA). The common leukocyte antigen CD14 and the CD45 expression were used for the "lymphogate" setup, as

previously described (Jeklova *et al.,* 2007a). The gates of each leukocyte type were adjusted with an isotype negative control. All the samples were processed in duplicate.

The total lymphocyte count was calculated as the product of the WBC count and the lymphocyte percentage, and of the lymphocyte subset counts and percentages, as described by Hulstaert *et al.* (1994).

2.4. Ultrasound measurements

The PFT of does was measured on 14 dpp by ultrasounds. Previously, fur was removed from the thoracic and lumbar vertebrae areas by shearing to improve image retrieval. Animals were placed in an immobilizing box (150 mm × 370 mm × 150 mm) during ultrasound measurement, and ultrasound gel was applied to the scanning area. The probe was always placed in the same position to obtain a repeatable transversal section of perirenal fat at 3 cm in front of the space between the second and third lumbar vertebrae. Images were obtained with an ultrasound unit (JustVision 200 'SSA-320A' real-time machine; Toshiba; Medical Systems Co., Ltd, Tokyo, Japan) equipped with a micro-convex electronic transducer of multi-frequency (5.0, 6.0 and 7.0 MHz; PVG-681S) and an image analyser software to determine distances. The average of the left- and right-side PFT was used for further calculations.

2.5. Statistical analysis

Data about lymphocyte populations in the blood of the rabbit does at first parturition were analysed using a general linear model (PROC GLM; Statistical Analysis System, 2002), with a model including only the animal type as fixed effect. To analyse the evolution of the lymphocyte populations in the blood of rabbit does after the first parturition, a mixed model (PROC MIXED; Statistical Analysis System, 2002) was used according to a repeated measures design, which takes into account the variation between animals and the covariation within them. Covariance structures were objectively compared using the most severe criteria (Schwarz Bayesian criterion), as suggested by Littell *et al*. (1998). The model included the animal type (AT: LP, V16 and V36), housing (H: CC or CH), the control day (D: first parturition, 4 dpp, 10 dpp and second parturition) and their interactions as fixed effects. The data from the control at the first partum were used as covariates within genetic lines (X_{ijklm}), where β was the regression of Y on the covariate. The random terms in the model included a permanent effect of each animal (p) nested to animal type and housing, and the error term (e).

$$y_{ijklm} = AT_i + H_j + D_k + AT_i \times H_j + AT_i \times D_k + H_j \times D_k + AT_i \times H_j \times D_k + \beta X_{ijklm} + p_l + e_{ijklm}$$

Finally, in order to test the relationship between the lymphocyte populations of rabbit does on 10 dpp with both feed intake during lactation week 2 and PFT on 14 dpp of females, Pearson's correlation coefficients (ρ) were obtained using PROC CORR of the Statistical Analysis System (2002).

3. STUDY DESIGN III

3.1. Animals

A total of 64 rabbit females from two different genetic lines (31 and 33 from V and LP, respectively) at second weaning were used in the present trial, coming from an initial group of 132 females. The V line has been selected for litter size at weaning for 31 generations, using as selection criterion the best linear unbiased prediction (BLUP) under a single-trait repeatability animal model (Estany *et al.,* 1989; García and Baselga, 2002a). The LP line was founded by selecting females from commercial farms showing an extreme longevity and an average life-time prolificacy per partum close to the average of the Spanish commercial population: i.e. at least 25 litters, with a minimum average litter size per partum of 7.5 kits born alive, as described by Sánchez *et al.* (2008). After the foundation this line has been selected for litter size at weaning for six generations.

The Committee of Ethics and Animal Welfare of the Universitat Politècnica de Valencia approved this study. All the animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005).

3.2. Experimental procedure

Throughout the experiment, rabbit females were housed in a conventional housing (with light alternating cycle of 16 light hours and 8 dark hours, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5ºC, respectively), using individual cages (700 × 500 × 320 mm) provided with a nest for the litter from 28th day of gestation. Animals were *ad libitum* fed with a commercial diet for reproductive rabbit does (218 g acid detergent fibre and 174 g crude protein per kg of dry matter; Cunilactal, Nutreco) throughout the whole experiment.

After first parturition litters of 132 females (60 from the V line and 72 from the LP line) were standardized to nine kits. A total of 43 females from both lines were successfully artificially inseminated (AI) at day 4 post first partum (PP), while the other 89 females were AI after first weaning (PW; day 30 post first partum). At second parturition, litter size was standardized to 9 kits in all PP females (PP9), and to 5 or 9 kits for PW females (PW5 and PW9, respectively). Therefore within each line, three experimental groups with different levels of productive effort until second weaning were obtained: high (PP9), short recovery time after first post-weaning and high litter size at second lactation (9 and 11 does for lines V and LP, respectively); medium (PW9), long recovery time after first post-weaning and high litter size at second lactation (11 does from each line); and low (PW5), long recovery time after first post-weaning and low litter size at second lactation (11 does from each line). Females were not mated during the second lactation to avoid heterogeneity.

3.3. Performance traits

To evaluate the possible correlation between the previous energy balance and the immunological response of females at second weaning, body weight (BW), perirenal fat thickness (PFT) and estimated body energy (EBE) at day 0, 10 and 30 post second parturition were recorded. The PFT of does was measured by ultrasound to evaluate body condition, as described by Pascual *et al.* (2000, 2004). The average of the left- and right-side

PFT was used for further calculations. The estimated body energy (EBE) content of does was determined from BW and PFT of does, using the equations proposed by Pascual *et al.* (2004) for body energy estimation at different physiological stages.

3.4. LPS challenge

An acute phase response was induced according to Saitoh *et al.* (2000), by lipopolysaccharide (LPS) challenge at day 30 post second parturition. LPS from *Escherichia coli* (serotype 0111:B4, L2630, Sigma Chemical Company, St. Louis, MO, USA) was dissolved in saline (0.25 mg/mL) and injected by marginal ear vein (50 µg/kg). Rectal body temperatures were measured (digital thermometer Citizen CT561C) and blood samples were collected from the central ear artery at 0, 1.5, 3, 6, 24 and 48 h after the LPS inoculation (11:00 a.m.) using vacuum tubes with EDTA. Plasma was obtained by centrifugation (3000×G, 10 min) at 4 ºC and stored at -80 ºC until analysis for glucose, non-esterified fatty acids (NEFA), haptoglobin and C-reactive protein.

3.5. Plasma analyse

Blood plasma glucose was determined according to standard procedures (Siemens Diagnostics® Clinical Methods for ADVIA 1650). NEFA were determined using the Wako, NEFA C ACS-ACOD assay method. Haptoglobin was determined chemically due to its ability to bind to haemoglobin, Phase ™, Tridelta Developments, Wicklow, Ireland. All analyses were performed using an autoanalyzer, ADVIA 1650® Chemistry System (Siemens Medical Solutions, Tarrytown, NY 10591, USA). The intra assay variabilities were in all instances below 2 % (CV); inter assay variation were in all instances below 4.5 % (CV).

Rabbit C-reactive protein was analysed by a commercial ELISA assay (Life Diagnostics, Inc., West Chester, PA 19380, USA). Manufacturers' instructions were followed. Intra- and inter assay variation were below 8%.

3.6. Statistical analysis

3.6.1. Rectal temperature and plasma traits data

To analyse the evolution of corporal temperature and blood plasma traits with time after LPS infusion, a mixed model (PROC MIXED; Statistical Analysis System, 2002) was fitted, accounting for the repeated measures design in the data that takes into account the variation between animals and covariation within them. Covariance structures were objectively compared using the most severe criteria (Schwarz Bayesian criterion), as suggested by Littell *et al.* (1998). The model included the time (0, 1.5, 3, 6, 24 and 48 h), and their interactions with the genetic type (LP, and V) and the group (PP9, PW5 and PW9) to gather differences in the evolution of the traits in function of these main effects. Randoms terms in the model included a permanent effect of each animal (p) and the error term (e). Contrast tests were also performed at each time to define punctual differences between genetic types and groups. To test the relationship between the previous performance traits during second lactation and both the rectal temperature and plasma traits at maximum response time after LPS challenge, Pearson's correlation coefficients (ρ) were obtained using PROC CORR of the Statistical Analysis System (2002).

3.6.2. Survival after the immunological challenge

Cumulative mortality of rabbit does after a LPS induced challenge at second weaning was firstly analysed by a χ^2 test using the PROC GENMOD of the Statistical Analysis System (2002).

The survival ability of the different females was also assessed using survival analysis techniques, to evaluate the effect of plasma traits on female survival. Females having a LCA equal to 48 h were assumed to have a censored record for time until death. The following proportional hazard model was fitted

$$h_{ik}\left(t \mid \mathbf{x}'_i(t)\right) = h_0(t)_k \times \exp\{\mathbf{x}'_i(t)\mathbf{b}\}$$

where $\mathbf{h_{ik}(t|x'_i(t))}$ is the hazard associated to the animal i at time t, $\mathbf{h_0(t)_k}$ is a baseline hazard function of time t; $\mathbf{x'_i(t)}$ is an incident vector for animal i relating covariates to the observations; **b** is a vector with the effect of covariates (C-reactive protein, glucose, NEFA haptoglobin and temperature). It was decided to use a stratified model in order to avoid the constraint of proportionality between hazards across levels of the combination genetic type by group. The presented proportional hazard model was fitted assuming the Cox likelihood and implemented using the Survival Kit software.

The survival function for six animals, one in each level of the interaction genetic type×group was predicted, for these animals the covariates in $\mathbf{x'_i(t)}$ were set to their mean value. An estimate of the log-hazard between two levels genetic type×group in a given time could be obtained from these predictions. At a given time **(t)** the survival function for an animal in a given combination genetic type by group L1 (for example, V in PW9) could be named $\hat{S}_{L_1}(t_t)$; for another animal in other combination L2 (for example LP in PW9) it will be $\hat{S}_{L_2}(t_t)$. At this time the proportionally between hazards holds, thus

$$\hat{h}_{L_1}(t_t) = \hat{h}_{L_2}(t_t) \times \gamma_{L1-L2,t}$$

where, $\gamma_{L1-L2,t}$ is the hazard ratio between L1 and L2 at time t (times more likely to die a L1 than a L2 female at time t); being $\theta_{L1-L2,t}$ the log-hazard ratio ($\log(\gamma_{L1-L2,t})$). Given the relationships between hazard and survival functions $\hat{S}_{0,L_1}(t_t) = \hat{S}_{0,L_2}(t_t)^{\gamma_{L1-L2,t}}$. In order to obtain a measurement on the uncertainty for the estimated differences between genetic types in each group, 10,000 bootstrap samples of the data set were obtained and for each model fitted, determining log-hazard ratios for each genetic type×group combination. Each bootstrap sample consisted of a random extraction with reposition from the original data set of as many records as the original data set had (64), in this way for each replication one particular record could be present several times and others will not appear, simulating random repetitions of a given experiment. The estimated effect for each level of the combination genetic type×group was the average across replicates, while its error was the standard deviation across replicates.

RESULTS

D. RESULTS

1. STUDY I

Tables 8a, b present the means, standard deviations and coefficients of variation for peripheral blood lymphocyte populations and ultrasound PFT measurements of rabbit does for all animals and in terms of their weaning days. Lymphocyte populations were characterized by higher $CD5^+$ T lymphocytes counts (1191 × 10^6/L) than B lymphocytes counts (96 × 10^6/L), which became more variable later among individuals (CV=75%). Very high variability was noted in the change of PFT during lactation (CV=1250%), mainly in the 42D does (CV=2589%).

Counts of total, $CD5^+$, $CD4^+$ and $CD8^+$ lymphocytes were significantly higher in the 28D than in the 42D rabbit does (P=0.003, P=0.002, P=0.016 and P=0.042, respectively). Differences in the evolution of total lymphocytes during the study period were detected in accordance with reproductive management rhythm (**Figure 58**). While no differences were seen in the 42D does, increased total lymphocytes at weaning (28 dpp) and next partum (42 dpp) were observed in the 28D does. Compared with the 42D does, 28D does presented a higher $CD5^+$ lymphocytes count on 16 dpp and 28 dpp due to increased $CD4^+$ and $CD8^+$ populations (only statistically significant on 28 dpp; P<0.05). At weaning, 28D females also presented higher counts of total, B, $CD5^+$, $CD4^+$ and $CD8^+$ lymphocytes than their 42D counterparts. These differences were not significant 1 week later.

Table 8a. Means, standard deviations and coefficients of variation for the lymphocyte population and ultrasound measurements of the perirenal fat thickness (PFT) of multiparous rabbit does.

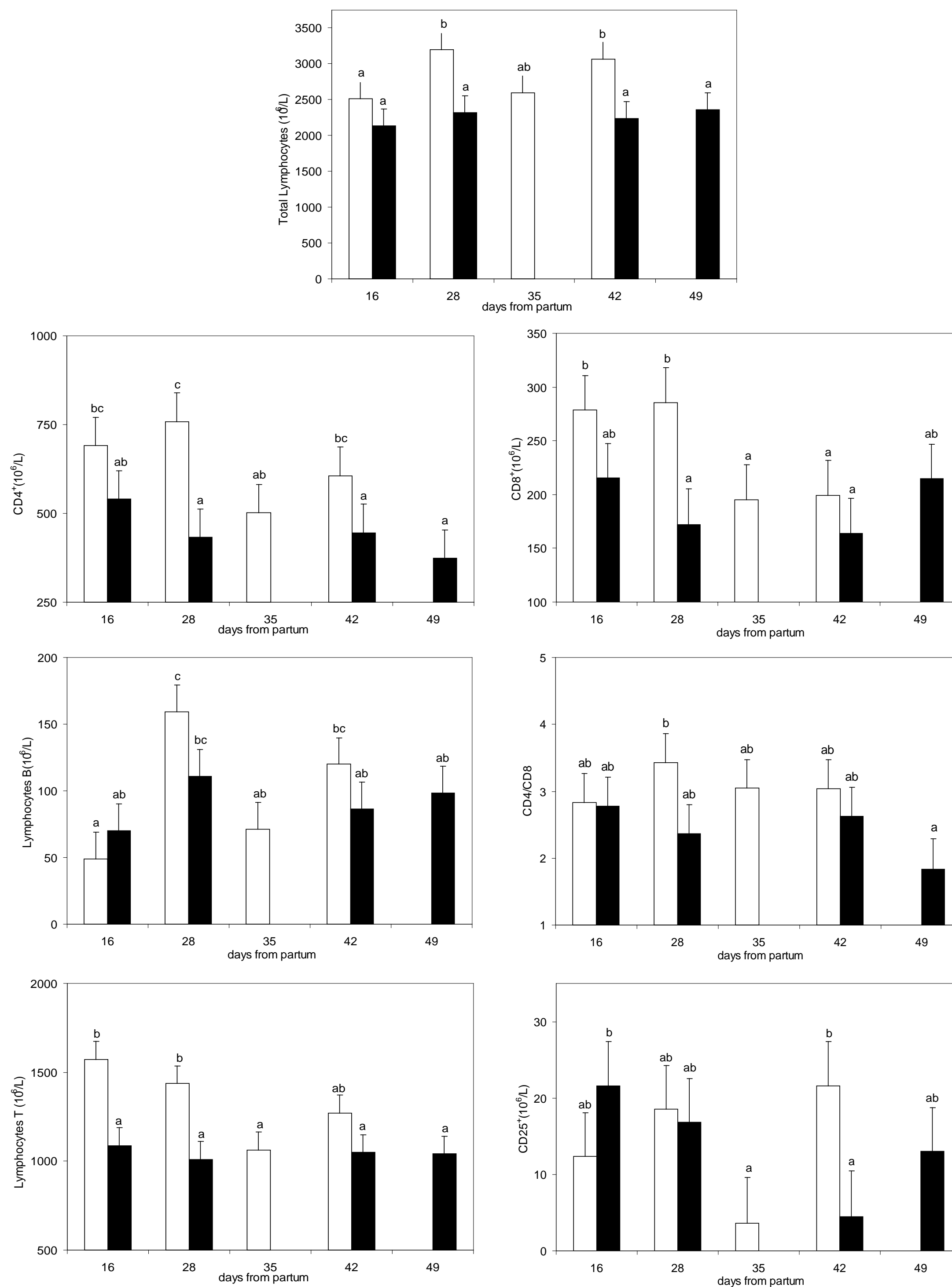
Variable	Description	No.[1]	Mean	SD[2]	Minimum	Maximum	CV(×100)[3]
All the females							
|Lym|	Lymphocytes $CD45^+CD14^-$(10^6/L)	88	2554	819	943	4566	32.08
|LymB|	B Lymphocytes (10^6/L)	88	96	72	0	339	74.88
|$CD5^+$|	$CD5^+$ T Lymphocytes (10^6/L)	88	1191	380	473	2262	31.94
|$CD4^+$|	$CD4^+$ (10^6/L)	88	543	283	85	1268	52.22
|$CD8^+$|	$CD8^+$ (10^6/L)	87	216	112	38	580	52.00
|$CD25^+$|	$CD25^+$ (10^6/L)	86	14	19	0	91	136.99
|$CD4^+$|/|$CD8^+$|		87	2.75	1.46	0.56	7.15	53.09
LymB	B Lymphocytes (%)	88	3.75	2.39	0	10.74	63.68
$CD5^+$	$CD5^+$ T Lymphocytes (%)	88	48.48	13.39	21.42	83.24	27.61
$CD4^+$	$CD4^+$ (%)	88	47.86	17.23	10.55	78.71	36.00
$CD8^+$	$CD8^+$ (%)	87	17.57	5.49	5.15	30.85	31.23
$CD25^+$	$CD25^+$ (%)	86	1.20	1.82	0	10.95	152.46
$CD4^+$/$CD8^+$		87	2.96	1.49	0.61	8.32	50.22
UP	PFT at partum (mm)	22	4.92	0.37	4.10	5.60	7.60
∆UPW	Partum to weaning PFT change (mm)	22	–0.04	0.51	–1.10	1.10	-1250.21

[1] *No.*: Number of observations.
[2] *SD*: Standard deviation.
[3] *CV*: Coefficient of variation.

Table 8b. Means, standard deviations and coefficients of variation for the lymphocyte population and ultrasound measurements of the perirenal fat thickness (PFT) of multiparous rabbit does in terms of their weaning age.

Variable	Description	No.[1]	Mean	SD[2]	Minimum	Maximum	CV(×100)[3]
Weaning at 28 days							
|Lym|	Lymphocytes $CD45^{+}CD14^{-}$(10^6/L)	44	2843	842	1535	4566	46.66
|LymB|	B Lymphocytes (10^6/L)	44	100	80	0	339	80.47
|$CD5^{+}$|	$CD5^{+}$ T Lymphocytes (10^6/L)	44	1335	400	632	2262	29.94
|$CD4^{+}$|	$CD4^{+}$ (10^6/L)	44	639	296	196	1268	46.46
|$CD8^{+}$|	$CD8^{+}$ (10^6/L)	44	240	123	78	580	51.48
|$CD25^{+}$|	$CD25^{+}$ (10^6/L)	43	14	19	0	85	130.19
|$CD4^{+}$|/|$CD8^{+}$|		44	3.09	1.58	0.56	7.15	51.14
LymB	B Lymphocytes (%)	44	3.51	2.61	0	10.74	74.34
$CD5^{+}$	$CD5^{+}$ T Lymphocytes (%)	44	49.08	15.39	21.42	83.24	31.36
$CD4^{+}$	$CD4^{+}$ (%)	44	50.24	13.90	12.79	77.75	27.68
$CD8^{+}$	$CD8^{+}$ (%)	44	17.09	5.55	5.15	30.85	32.46
$CD25^{+}$	$CD25^{+}$ (%)	43	0.91	1.07	0	5.33	117.64
$CD4^{+}$/$CD8^{+}$		44	3.32	1.55	0.61	8.32	46.67
UP	PFT at partum (mm)	11	4.80	0.37	4.10	5.30	7.71
ΔUP28d	Partum to 28 days PFT change (mm)	11	–0.06	0.57	–1.1	1.10	–897.98
Weaning at 42 days							
|Lym|	Lymphocytes $CD45^{+}CD14^{-}$(10^6/L)	44	2265	691	943	3627	30.53
|LymB|	B Lymphocytes (10^6/L)	44	91.43	62.34	0	307	68.18
|LymT|	$CD5^{+}$ T Lymphocytes (10^6/L)	44	1046	300	473	1734	28.70
|$CD4^{+}$|	$CD4^{+}$ (10^6/L)	44	448	237	85	983	52.90
|$CD8^{+}$|	$CD8^{+}$ (10^6/L)	43	192	95	38	439	49.64
|$CD25^{+}$|	$CD25^{+}$ (10^6/L)	43	14	20	0	91	145.15
|$CD4^{+}$|/|$CD8^{+}$|		43	2.40	1.25	0.77	5.95	52.02
LymB	B Lymphocytes (%)	44	3.99	2.15	0	9.27	53.81
LymT	$CD5^{+}$ T Lymphocytes (%)	44	47.87	11.17	27.29	73.14	23.34
$CD4^{+}$	$CD4^{+}$ (%)	44	45.48	19.90	10.55	78.71	43.74
$CD8^{+}$	$CD8^{+}$ (%)	43	18.05	5.44	5.15	30.53	30.15
$CD25^{+}$	$CD25^{+}$ (%)	43	1.48	2.33	0	10.95	156.99
$CD4^{+}$/$CD8^{+}$		43	2.60	1.35	0.87	6.89	51.85
UP	PFT at partum (mm)	11	5.05	0.35	4.60	5.60	6.89
ΔUP42d	Partum to 42 days PFT change (mm)	11	–0.02	0.47	–0.70	0.70	–2589.30

[1] *No.*: Number of observations.
[2] *SD*: Standard deviation.
[3] *CV*: Coefficient of variation.



[a,b,c] The least square means not sharing the same superscript significantly differ at $P<0.05$.

Figure 58. Effect of reproductive rhythm (weaning at 28 □ or 42 ■ days) on the evolution of lymphocytes populations ($\times 10^6$/L) in the peripheral blood of multiparous rabbit does.

Table 9 shows the simple correlation coefficients between the body condition traits and lymphocyte population counts of the 42D rabbit does (no relevant correlation was found in the 28D rabbit does). The greater the PFT at partum, the higher the total and B lymphocytes

counts on 16 dpp. Furthermore, the greater the PFT at weaning or the lesser the PFT losses during lactation, the higher the B and $CD5^+$ lymphocyte counts 1 week later. In addition, the PFT change during lactation negatively correlated with the $CD4^+/CD8^+$ ratio.

The only statistically significant difference observed for young rabbits was for the $CD4^+$ lymphocyte count, which was higher at weaning for those weaned at 28 than at 42 days (**Table 10**).

Table 9. Simple correlation coefficients[a] between PFT thickness during lactation and lymphocyte populations in the peripheral blood of multiparous rabbit does weaned at 42 days[b] (n=11).

Population[c]	Time	UP[c]	U42d[c]	ΔUP42d[c]
$\|LymCD45^+ CD14^-\|$	16days	$+0.6477^*$		
$\|LymB\|$	16days	$+0.8215^{***}$		
	49days		$+0.6030^*$	$+0.7030^{**}$
$\|CD5^+\|$	49days		$+0.6875^{**}$	$+0.7901^{**}$
$\|CD4^+\|/\|CD8^+\|$	42days			-0.6646^*
	49days			-0.7217^{**}

[a] Only relevant and significant correlations are presented: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.
[b] No relevant simple correlations for the data from multiparous rabbit does weaned at 28 days were obtained.
[c] Abbreviations as in Table 8a, b.

Table 10. Effect of reproductive rhythm (weaning at 28 or 42 days) on lymphocyte populations in the peripheral blood of young rabbits at weaning (mean ± SE).

Population[1]	No.	Weaning age		*P*-value
		28 d	42 d	
$\|LymCD45^+ CD14^-\|$ (10^6/L)	41	1713 ± 163	1635 ± 152	0.7317
$\|LymB\|$ (10^6/L)	41	66.0 ± 14.5	97.7 ± 13.5	0.1172
$\|CD5^+\|$ (10^6/L)	41	749 ± 100	735 ± 93	0.9197
$\|CD4^+\|$ (10^6/L)	42	409 ± 41	255 ± 39	0.0095
$\|CD8^+\|$ (10^6/L)	42	181 ± 24	159 ± 22	0.5039
$\|CD25^+\|$ (10^6/L)	38	6.25 ± 3.19	8.68 ± 2.9	0.4794
$\|CD4^+\|/\|CD8^+\|$	40	2.32 ± 0.24	1.91 ± 0.22	0.2213
LymB (%)	41	4.39 ± 1.26	6.81 ± 1.12	0.1678
$CD5^+$ (%)	41	48.30 ± 7.05	43.76 ± 6.25	0.6348
$CD4^+$ (%)	42	55.75 ± 5.02	45.89 ± 4.62	0.1642
$CD8^+$ (%)	42	24.79 ± 2.05	25.49 ± 1.89	0.8031
$CD25^+$ (%)	38	0.44 ± 0.48	1.48 ± 0.44	0.1265
$CD4^+/CD8^+$	40	2.26 ± 0.19	1.77 ± 0.16	0.0691

[1] Abbreviations as in Table 8a, b.
SE: Standard error.

Some significant correlations were detected between the lymphocyte populations of females and young rabbits (**Table 11**). In particular, females' $CD5^+$ lymphocyte count positively correlated with young rabbits' $CD5^+$, $CD4^+$ and $CD8^+$ lymphocyte counts, but negatively correlated with young rabbits' $CD25^+$ lymphocyte count.

Table 11. Simple correlation coefficients[1] between lymphocyte populations in the peripheral blood of young rabbits at weaning and those of multiparous rabbit does on different days (n=22).

	Lymphocyte populations of pups at weaning[2]				
Lymphocyte population of females[2]	**|Lym|**	**$\|CD5^+\|$**	**$\|CD4^+\|$**	**$\|CD8^+\|$**	**$\|CD25^+\|$**
$\|LymCD45^+CD14^-\|$					–0.3343*
|LymB|		+0.3534*			
$\|CD5^+\|$		+0.3740**	+0.3505*	+0.3418*	–0.3958**
$\|CD4^+\|/\|CD8^+\|$	+0.3196*		+0.3233*		

[1] Only relevant and significant correlations are presented: * P<0.05; ** P<0.01; *** P<0.001.
[2] Abbreviations as in Table 8a, b.

2. STUDY II

Table 12 shows the effect of animal type and housing on lymphocytes populations. Since many interactions between the main factors were found, a three-way interaction is represented in **Figures 59a, b, c** and **60a, b, c**. As seen in **Table 12**, housing under heat stress conditions resulted only in a significant reduction of the B lymphocytes counts in female rabbits (−34 ± 14 × 10^6/L; P<0.05). Yet the blood counts of the majority of lymphocytes populations (total, T $CD5^+$, $CD4^+$ and $CD8^+$) showed the highest values at first parturition (on average +443, +252, +113 and +73 × 10^6/L if compared to the remaining control days; P<0.01), while the B lymphocytes count was significantly lower at second parturition (−61 ± 16 × 10^6/L; P<0.05).

Table 12. Effect of animal type and housing of rabbit does on lymphocyte populations (10^6/L) in peripheral blood from the first to the second parturition.

	Animal type (G)[1]			Housing (H)[2]	
	LP	V16	V36	CC	CH
Total Lymphocytes ($CD45^+ CD14^-$)	2816 ± 133^b	2969 ± 119^b	2467 ± 125^a	2754 ± 102	2748 ± 106
B Lymphocytes	130.8 ± 10.9^{ab}	166.5 ± 10.4^b	106.9 ± 10.6^a	117.7 ± 9.2	151.7 ± 9.2
$CD5^+$ T Lymphocytes	1312 ± 67	1381 ± 57	1268 ± 61	1334 ± 49	1306 ± 49
$CD4^+$	748.5 ± 49.2	790.1 ± 41.7	690.5 ± 46.7	751.9 ± 37.4	734.2 ± 35.6
$CD8^+$	323.4 ± 18.5	338.5 ± 16.9	332.3 ± 16.8	334.3 ± 14.0	328.6 ± 14.3
$CD25^+$	52.15 ± 4.24^b	40.02 ± 3.76^{ab}	31.17 ± 3.96^a	43.46 ± 3.29	38.77 ± 3.25

	P-value						
	G	H	D[3]	G×H	G×D	H×D	G×H×D
Total Lymphocytes ($CD45^+ CD14^-$)	0.3555	0.9701	0.0240	0.9242	0.0240	0.7180	0.0388
B Lymphocytes	0.0536	0.0157	0.0002	0.0222	0.4269	0.2581	0.0212
$CD5^+$ T Lymphocytes	0.6851	0.6824	0.0001	0.8511	0.0074	0.7905	0.1058
$CD4^+$	0.8860	0.7244	0.0001	0.8349	0.0271	0.8050	0.5792
$CD8^+$	0.2685	0.7751	0.0034	0.8106	0.1566	0.7129	0.3335
$CD25^+$	0.2962	0.3179	0.0377	0.3105	0.0859	0.2293	0.2647

[1] *Animal type*: LP line constituted by selection for hyperlongevity and reproductive criteria; V16 and V36, populations selected for litter size at weaning for 16 and 36 generations.
[2] *Housing*: CC, climatic chamber; CH, conventional housing.
[3] *D*: control day (4 and 10 days post first partum and second partum).
[a,b]: Means in the same row not sharing alphabets significantly differed (P<0.05) for animal type.

The V36 population rabbit did not present a lower number of total lymphocytes (**Table 12**) than those of the V16 population (−502 ± 173 × 10^6/L; P<0.01) and the LP line (−349 ± 172 × 10^6/L; P<0.05). This scenario relates mainly to a drop in this cellular population in the V36 females housed in CH on 10 dpp and at the second parturition, when differences between V36 and V16 females were significant (**Figure 59a**). In LP females, the number of total lymphocytes lowered from the first parturition to 4 dpp (−1205 ± 421 × 10^6/L, P<0.001; **Figure 59a**). If compared to CH, the total counts at the second parturition in CC significantly increased for LP females (+947 ± 476 × 10^6/L; P<0.05), but lowered for V16 females (−808 ± 403 × 10^6/L; P<0.05). The difference between LP and V36 females reached the level of significance (**Figure 59a**).

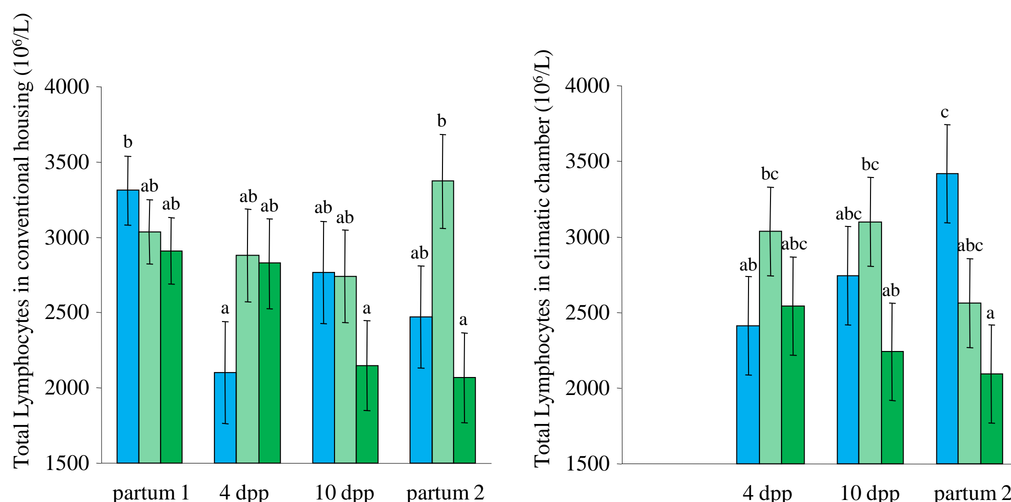


Figure 59a. Effect of animal type (LP ■, V16 ■ and V36 ■) on the evolution of total lymphocytes ($\times 10^6$/L) in the peripheral blood of rabbit does when housed in a conventional environment [left figure] or in a climatic chamber under heat stress conditions [right figure]. The LP line was constituted by hyperlongevity and reproductive criteria selection; V16 and V36 populations were selected for litter size at weaning for 16 and 36 generations. [a,b,c] The means for each environment, which do not share a superscript in the same figure, were significantly different (P<0.05). Error bars correspond to the standard error for each least square mean.

The V36 population females presented lower B lymphocytes counts than the V16 ones (−60 ± 15 × 10^6/L; P<0.001), while LP females showed intermediate counts (**Table 12**). These results relate mainly with the high counts recorded on 4 dpp and 10 dpp for V16 in CH, which were not detected in CC because of the significant reduction noted (−122 and −129 ± 39 × 10^6/L on 4 dpp and 10 dpp, respectively, P<0.01; **Figure 59b**).

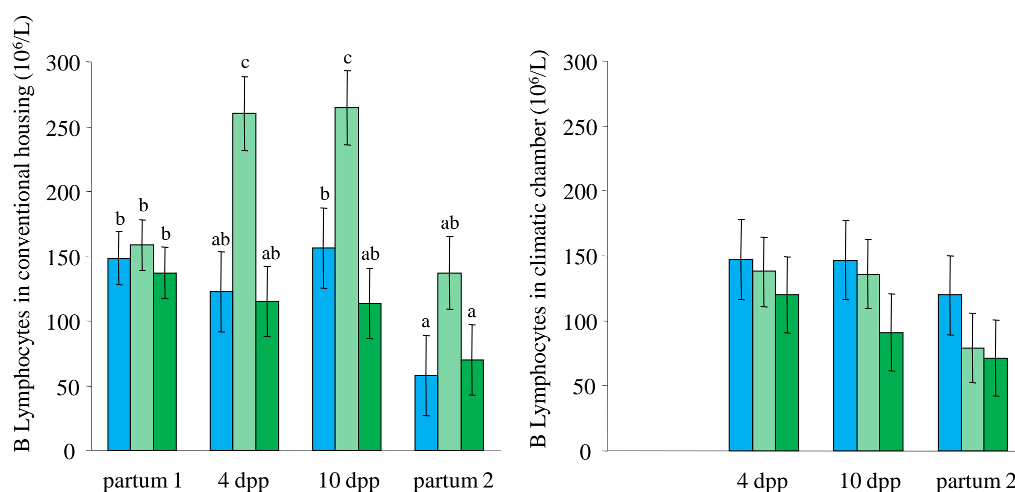


Figure 59b. Effect of animal type (LP ■, V16 ■ and V36 ■) on the evolution of B lymphocytes ($\times 10^6$/L) in the peripheral blood of rabbit does when housed in a conventional environment [left figure] or in a climatic chamber under heat stress conditions [right figure]. The LP line was

constituted by hyperlongevity and reproductive criteria selection; V16 and V36 populations were selected for litter size at weaning for 16 and 36 generations. [a,b,c] The means for each environment, which do not share a superscript in the same figure, were significantly different (P<0.05). Error bars correspond to the standard error for each least square mean.

At the second parturition in CC, the LP line animals displayed higher counts than those of the V line (not statistically significant, on average +46 ± 40 × 10^6/L; P>0.10). A positive relationship was found between B lymphocytes in blood on 10 dpp with feed intake during lactation week 2 (r=+0.51; P<0.001) and PFT on 14 dpp (r=+0.40; P<0.001).

In CH, the T $CD5^+$ lymphocytes counts followed a similar pattern to those of total lymphocytes, with higher counts for V16 females than those for LP and V36 females at second parturition (on average +30%, P<0.05; **Figure 59c**).

As described for total lymphocytes, the counts recorded at the second parturition in CC, if compared to CH, were higher for LP females, but lower for V16 females. However, differences were not statistically significant (LP line: +355 ± 204 × 10^6/L; P<0.10; V16: −295 ± 189 × 10^6/L; P>0.10). Similarly to the observations made for B lymphocytes at the second parturition in CC, the LP line animals had higher counts than those from both the V line populations (not statistically significant; on average +260 ± 194 × 10^6/L; P>0.10).

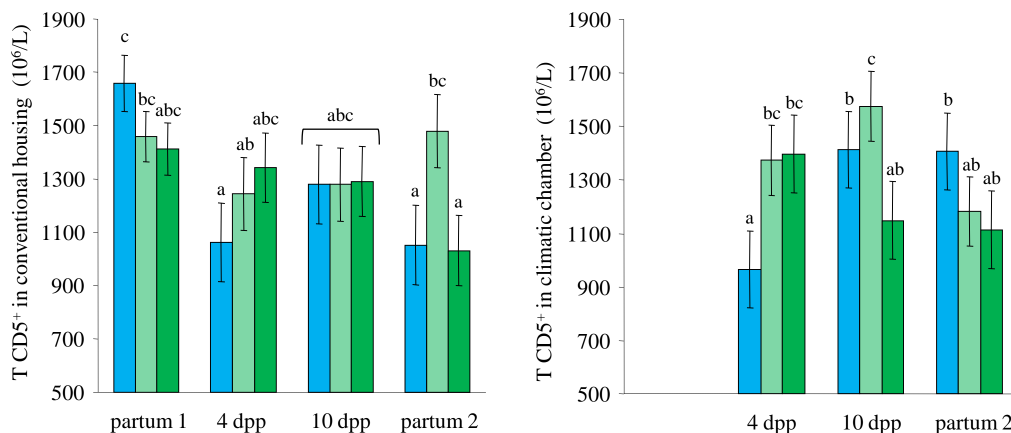


Figure 59c. Effect of animal type (LP ■, V16 ■ and V36 ■) on the evolution of T $CD5^+$ lymphocytes (×10^6/L) in the peripheral blood of rabbit does when housed in a conventional environment [left figure] or in a climatic chamber under heat stress conditions [right figure]. The LP line was constituted by hyperlongevity and reproductive criteria selection; V16 and V36 populations were selected for litter size at weaning for 16 and 36 generations. [a,b,c] The means for each environment, which do not share a superscript in the same figure, were significantly different (P<0.05). Error bars correspond to the standard error for each least square mean.

The counts of lymphocytes $CD4^+$ and $CD8^+$ fitted the pattern described for T $CD5^+$ lymphocytes and for the changes relating to animal type or housing (**Figure 60a** and **b**). However, $CD25^+$ lymphocytes counts followed a dissimilar pattern because they were higher in LP females than in V36 females (average +20.98 ± 5.80 × 10^6/L; P<0.001; **Table 12**). This was due mainly to the differences noted in CH from 4 dpp onwards (**Figure 60c**). When compared to CH, a significant increase in $CD25^+$ counts was detected in CC for V16 females on 10 dpp (+47.6 ± 15.7 × 10^6/L; P<0.01).

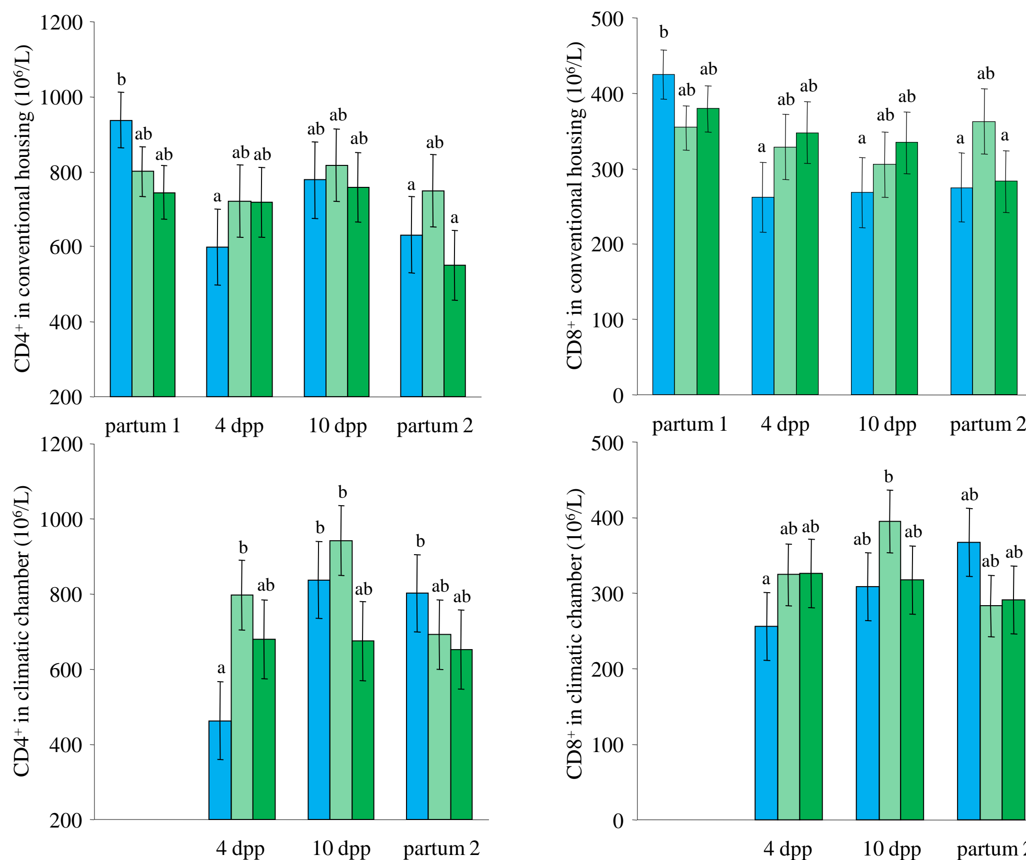


Figure 60a, b. Effect of animal type (LP, V16 and V36) on the evolution of lymphocytes (**a**) $CD4^+$ [left figures], (**b**) $CD8^+$ ($\times 10^6$/L) [right figures] in the peripheral blood of rabbit does when housed in a conventional environment [upper figures] or in a climatic chamber under heat stress conditions [lower figures]. The LP line was constituted by hyperlongevity and reproductive criteria selection; V16 and V36 populations were selected for litter size at weaning for 16 and 36 generations. [a,b,c] The means for each environment, which do not share a superscript in the same figure, were significantly different (P<0.05). Error bars correspond to the standard error for each least square mean.

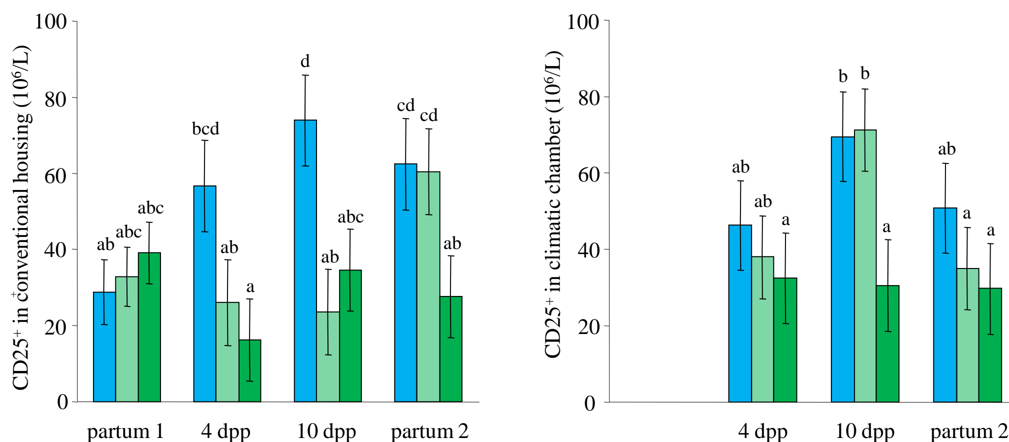


Figure 60c. Effect of animal type (LP, V16 and V36) on the evolution of T $CD25^+$ lymphocytes ($\times 10^6$/L) in the peripheral blood of rabbit does when housed in a conventional environment [left

figure] or in a climatic chamber under heat stress conditions [right figure]. The LP line was constituted by hyperlongevity and reproductive criteria selection; V16 and V36 populations were selected for litter size at weaning for 16 and 36 generations. [a,b,c] The means for each environment, which do not share a superscript in the same figure, were significantly different (P<0.05). Error bars correspond to the standard error for each least square mean.

3. STUDY III

3.1. Rectal temperature and plasma traits

As a result of the different genetic types and previous reproductive efforts used, females arrived at second weaning with relative differences in body condition (coefficient of variation (CV) from 8% to 11%), ranging in BW from 3.3 to 5.2 kg and PFT from 4.8 to 10.0 mm (**Table 13**). During the induced challenge, the concentrations of all the plasma metabolites showed high variability (CV from 34% to 152%).

Table 13. Means, standard deviations and coefficients of variation for the body condition traits and plasma metabolite concentrations measured.

Variable	No.	Mean	SD	Minimum	Maximum	CV
Body weight at weaning (g)	64	4076	341	3365	5155	8.35
Perirenal fat thickness at weaning (mm)	64	7.83	1.28	4.80	10.0	10.14
Estimated body energy at weaning (MJ/kg BW)	64	9.32	1.04	7.41	12.42	11.19
Rectal temperature (ºC)	339	40.5	0.8	38.4	42.0	1.97
Non esterified fatty acids (μeqv/L)	310	947	732	73	5061	77.35
Glucose (mM)	307	7.22	2.48	0.86	16.89	34.37
c-reactive protein (mg/L)	305	179	272	1.1	1057	152.4
Haptoglobin (mg/mL)	306	1.09	1.22	0.02	5.99	112.1

SD: standard deviation.
CV: coefficient of variation (%).

Time from challenge significantly affected all the variables analyzed (P<0.001). As seen in **Figure 61**, the evolution of rectal temperature of females after infusion was similar for both genetic types, with minor exceptions at one time point (P=0.09), with a sharp increase just after infusion (+1.45ºC at 3 h; P<0.001) to then return to normal ranges at 48 h post-infusion (pi). LP females showed a lower rectal temperature than V females (–0.3ºC; P<0.05) just at 6 h pi.

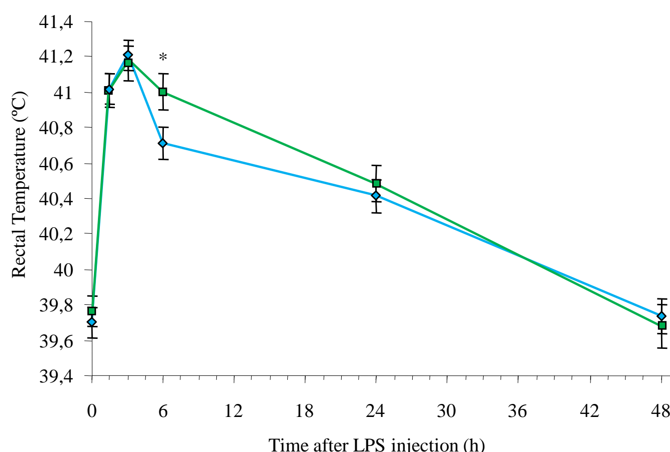


Figure 61. Evolution of rectal temperature in LP (—) and V (—) rabbit does after a LPS induced challenge at second weaning. * Significant differences between means at P<0.05.

The evolution of plasma NEFA concentration was highly affected by the group (P<0.001; **Figure 62**). PP9 females showed similar plasma NEFA concentrations for all the time-points after the challenge, but NEFA was highly increased in PW9 and PW5 groups at 24 (on av. +580 μeKv/L respect to PP9; P<0.05) and 48 h pi (on av. +1425 μeKv/L respect to PP9; P<0.001).

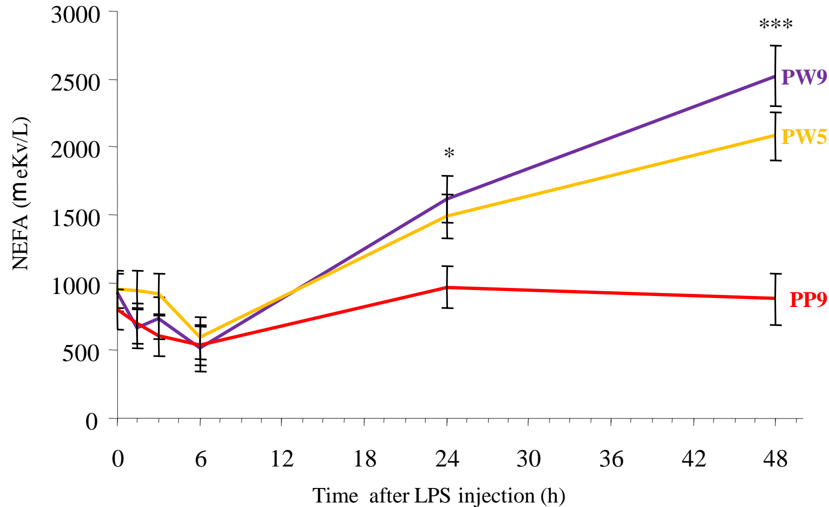


Figure 62. Evolution of plasma non-esterified fatty acids after a LPS induced challenge at second weaning, according to the previous reproductive effort (PP9, previous AI post-partum and 9 kits; PW9, previous AI post-weaning and 9 kits; and PW5, previous AI post-weaning and 5 kits). Significant differences between PW5 and PW9 with PP9 means at each time are presented as * P<0.05 or *** P<0.001.

Genetic type had no effect on the evolution of plasma NEFA levels after the challenge (P=0.709; **Figure 63a**). Glucose concentrations in plasma evolved in a similar way for both genetic types (P=0.077), reaching a peak after infusion (+2.5 mM at 1.5 h; P<0.001) and decreasing to a low at 24 h (–3.1 mM; P<0.001) before recovering to normal ranges at 48 h pi (**Figure 63b**). V females showed a higher plasma glucose content than LP females (+1.2mM; P<0.01) only at 1.5 h pi.

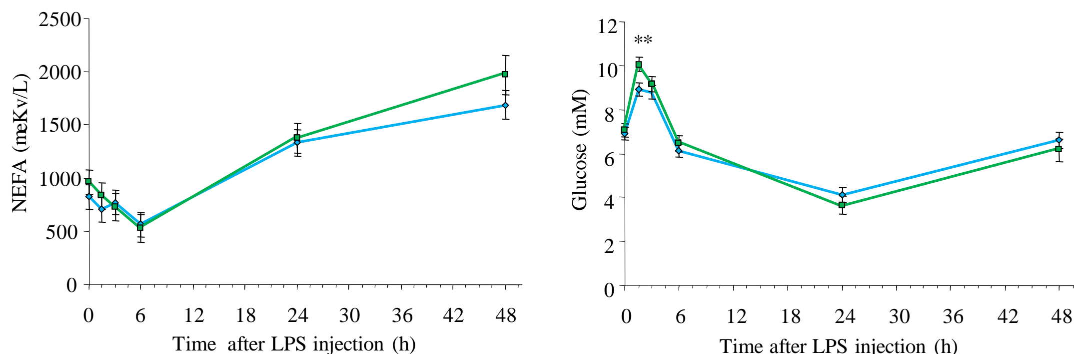


Figure 63a, b. Evolution of plasma concentrations of: (**a**) non-esterified fatty acids (NEFA) [left figure] and (**b**) glucose [right figure] in LP (—) and V (—) rabbit does after a LPS induced challenge at second weaning. Significant differences between means at ** P<0.01.

Both acute phase proteins measured showed a similar trend during the challenge (**Figures 63c** and **63d**). They were maintained at basal level until 6 h pi (on av. 38 mg/L and 0.47 mg/mL of C-reactive protein and haptoglobin, respectively), but showed important progressive increases from this time up to 48 h pi (on av. +685 mg/L and +2.89 mg/mL respect to the basal level, respectively). Differences between genetic types were found at 48h pi for the level of C-reactive protein (P=0.08) and haptoglobin in plasma (P<0.001). LP females had higher concentrations of haptoglobin at 24 h pi (+0.46 mg/mL; P<0.01), and of haptoglobin (+0.80 mg/mL; P<0.001) and C-reactive protein (+67 mg/L; P<0.10) at 48 h pi, than V females.

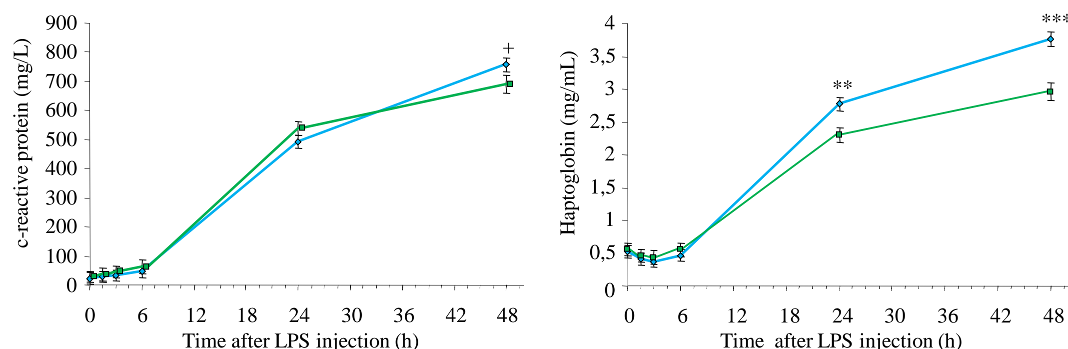


Figure 63c, d. Evolution of plasma concentrations of: (**c**) C-reactive protein [left figure] and (**d**) haptoglobin [right figure] in LP (—) and V (—) rabbit does after a LPS induced challenge at second weaning. Significant differences between means at $^{+}$P<0.10, **P<0.01, ***P<0.001.

Table 14 shows the linear relationships found between body condition traits of females before the induced challenge with the peak of the rectal temperature and plasma traits registered during the challenge. The greater the mobilization of reserves between 10 and 30 dpp (r=–0.34; P<0.05) the greater the rectal temperature measured at 3 h pi. The smaller the amount of PFT and EBE at 10 dpp the higher the plasma concentration of glucose at 1.5 h pi (r=–0.26 and –0.28, respectively; P<0.05). Finally, the lower the PFT at 0, 10 and 30 dpp the greater the plasma concentration of NEFAs recorded at 48 h pi (r=–0.35, –0.36 and –0.36; P<0.05).

Table 14. Correlation coefficients of some body condition traits with the rectal temperature (T) and plasma concentration on glucose, non-esterified fatty acids (NEFAs) and haptoglobin at maximum response time after LPS challenge.

	No.	PFT_{0d}	PFT_{10d}	PFT_{30d}	EBE_{10d}	$\Delta EBE_{10\text{-}30d}$
Rectal T_{3h}	56	+0.0456	+0.2573	–0.1211	+0.2069	-0.3384^{*}
$Glucose_{1.5h}$	59	–0.0897	-0.2574^{*}	–0.1544	-0.2778^{*}	+0.0987
$NEFA_{48h}$	32	-0.3508^{*}	-0.3612^{*}	-0.3611^{*}	–0.2023	–0.0472
$Haptoglobin_{48h}$	32	$+0.2950^{+}$	+0.0727	+0.0616	+0.0346	+0.0960

PFT: perirenal fat thickness.
EBE: estimated body energy.
Δ*EBE*: change of estimated body energy between 10 and 30 days.
Differences in the number of observation for each variable were due to the different number of animals alive at each sampling time: 59, 56 and 32 at 1.5, 3 and 48 h after LPS inoculation, respectively.

3.2. Survival after immunological challenge

As a consequence of the induced challenge, V females had a higher cumulative mortality up to 6 h pi than LP females (+20% at 3 and 6 h pi; P<0.05) and, although differences remained after that, they were not significant (**Figure 64**). Females from PP9 group presented a significantly (P<0.05) lower mortality rate at 6 h (5 vs. 20%) and 48 h pi (20 vs. 41%) with respect to the PW5 and PW9 groups.

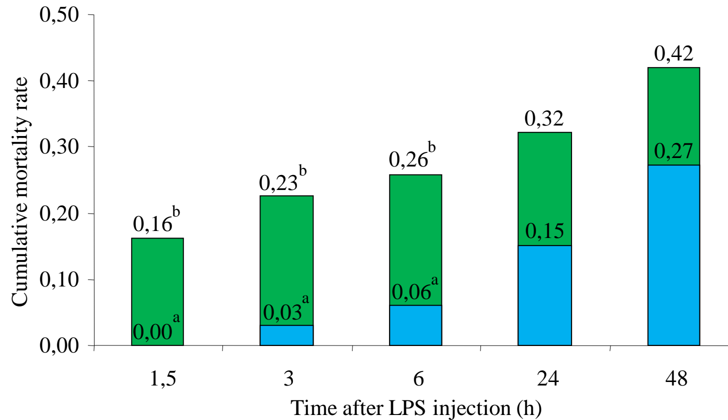


Figure 64. Cumulative mortality of LP (■) and V (■) rabbit does after a LPS induced challenge at second weaning. Means in a same time not sharing superscripts are different at P<0.05 (χ^2 test).

The ratios between the probabilities of culling associated with genetic type for the different treatment groups and across time are presented in the **Table 15**.

Table 15. Hazard ratio of death between different genetic types for each group (PP9, previous AI post-partum and 9 kits; PW9, previous AI post-weaning and 9 kits; and PW5, previous AI post-weaning and 5 kits) across time.

Genetic type contrast	Group	Time (h)	Hazard ratio[1]		
			Log_e(hazard)	SD Log_e(hazard)	Hazard ratio ($e^{Log_e(hazard)}$)
V-LP	PW9	0.5	12.02	5.64	166791.53
		1.5	12.02	5.63	166791.53
		3	1.57	8.13	4.79
		6	1.91	7.94	6.79
		24	1.91	7.94	6.79
		48	1.68	5.59	5.39
V-LP	PW5	0.5	12.36	3.33	232838.42
		1.5	12.36	3.54	232838.42
		3	12.36	3.54	232838.42
		6	12.36	3.54	232838.42
		24	1.48	8.01	4.41
		48	0.17	3.00	1.18
V-LP	PP9	0.5	0.00	0.00	1.00
		1.5	0.00	0.00	1.00
		3	0.00	0.00	1.00
		6	-11.40	7.81	0.00
		24	-1.09	8.75	0.34
		48	-1.09	8.75	0.34

SD: Standard deviation.

For females mated at weaning after the first cycle, a V female was 166,791 times more likely to die than an LP female until 1.5 h pi when the females were under treatment PW9. If they were under treatment PW5, the relative risk was also very high, 232,838 and it extended until 6 h pi. However, when females were mated at parturition after the first cycle (PP9), the risk was similar for both genetic types until 3 h pi. From 3-6 h pi, hazard differences in favour of LP females disappeared or even tended to reverse, there being a lower risk of death for the V line. Having observed extreme values for the relative risks between lines at given times, it is a consequence of a lack of animals dying at these times. The solution to avoid this issue would be to have a larger data set in order to give the chance of observing a die in those combinations of genetic type and group for which so far none deaths have been observed at that certain control times. In spite of this, the lack of dying animals from a specific genetic time in a given time might be an evidence of the lower risk of culling for this genetic type with respect to the other at that time.

To evaluate the role of the controlled blood plasma traits as mediators of the different survival rates associated with the different levels of the combinations between genetic type and treatment group, different stratified analyses were conducted (**Table 16**), where the base hazard function was assumed to be defined by the different component of the combination genetic type by group. When a unique baseline hazard function was considered, i.e. the effect of the genetic type and group were not considered, a significant association between NEFA plasma concentration and the hazard was found, being the estimate in this case 0.46×10^{-3}. Nearly the same estimate was obtained when the baseline hazard function was defined exclusively either by the genetic type or the treatment group, indicating that the effect of these factors it was not mediated throughout differences in NEFA levels. However, when both factors were used for defining the baseline hazard function the NEFA estimate of risk dropped to 0.39×10^{-3} and it became non-significant.

Table 16. Stratified analyses for the regression coefficients (b) of time dependent traits on the $\log_e$(hazard) of death or culling.

Model design	complete		without genetic type		without group		without genetic type nor group	
	b[1] ± SE	P-value	b ± SE	P-value	b ± SE	P-value	b ± SE	P-value
Rectal temperature (ºC)	0.227 ± 0.408	0.5785	0.305 ± 0.393	0.4380	0.518 ± 0.383	0.1760	0.508 ± 0.375	0.1757
NEFA[2] (µeqv/L)	$0.391\times10^{-3} \pm 0.231\times10^{-3}$	0.0897	$0.490\times10^{-3} \pm 0.231\times10^{-3}$	0.0309	$0.456\times10^{-3} \pm 0.194\times10^{-3}$	0.0188	$0.465\times10^{-3} \pm 0.192\times10^{-3}$	0.0156
Glucose (mM)	0.206 ± 0.119	0.0822	0.156 ± 0.110	0.1558	0.127 ± 0.109	0.2438	0.138 ± 0.109	0.2042
c-reactive protein (mg/L)	$0.104\times10^{-3} \pm 0.142\times10^{-2}$	0.4616	$0.558\times10^{-3} \pm 0.136\times10^{-2}$	0.6819	$0.763\times10^{-3} \pm 0.135\times10^{-2}$	0.5709	$0.629\times10^{-3} \pm 0.133\times10^{-2}$	0.6291
Haptoglobin (mg/mL)	–0.237 ± 0.326	0.4663	–0.116 ± 0.300	0.6988	–0.170 ± 0.315	0.5900	–0.107 ± 0.293	0.7151

[1] e^b: increase of the probability of death or culling of the animal per unit of trait increased.
[2] *NEFA*: Non-esterified fatty acid.

GENERAL DISCUSSION

E. GENERAL DISCUSSION

Effect of weaning age (28 vs. 42 dpp) on the peripheral blood lymphocyte populations of multiparous rabbit does and their litters.

Rabbits have been used as experimental models for years, and haematological parameters and lymphocyte subsets in different types of animals can be found in the bibliography: conventional or SPF (free of common rabbit pathogens) rabbits, neonatal to pubescent rabbits, primiparous rabbit does and adult rabbits (Wells *et al.,* 1999; Jeklova *et al.,* 2007a; Çetin *et al.,* 2009; Jeklova *et al.,* 2009). However to the authors' knowledge, there is no information about these parameters, mainly lymphocyte subpopulations, in adult rabbit does under field conditions.

The first objective of the thesis was to study the effect of two reproductive management rhythms on the lymphocyte subpopulations of multiparous rabbit does.

Several imbalances appear in the results obtained in the present study. The sum of the $CD5^{+}$ and B lymphocyte percentages is lower than 100, averaging 52% in both rabbit does and weaning rabbits. Jeklova *et al.* (2007a) referred to these non-detected cells as lymphocytes with pT^{-}, $CD4^{-}$, $CD8^{-}$, $CD79\alpha^{-}$ phenotypes that decreased with age, and constituted 45% and 16% of the total lymphocyte population in 1-day-old rabbits and 20-week-old rabbits, respectively. This discrepancy in the non-detected cells may relate with differences in age and the productive carrier of animals (present work: adult rabbit does at the end of their productive life; Jeklova *et al.* (2007a): pubescent rabbits), but also with the panels of antibodies used between both studies. Although the monoclonal antibody KEN-5 recognizes rabbit CD5-like molecules almost exclusively on T cells (Kotani *et al.,* 1993a,b), has also been reported that has an unusual limited reactivity compared to other authentic anti-CD5 antibodies (Pospisil *et al.,* 2009). The current difficulty for immunological research into rabbits lies in the still limited number of (commercially available) monoclonal antibodies that recognise the various lymphocyte subsets (Drouet-Viard and Fortun-Lamothe, 2002). In spite of this limitation, the comparison made between the two reproductive rhythms of present study is not invalidated.

The number of total lymphocytes in young weaning rabbits is lower than in reproductive rabbit does, which is in agreement with a gradual increase in lymphocytes during the previously described young rabbits' maturation (Jeklova *et al.,* 2007a, 2009). However, the mean values of the total lymphocytes in both rabbit does and weaning rabbits are lower than previously reported values (Wells *et al.,* 1999; Kim *et al.,* 2002b; Jeklova *et al.,* 2007a, 2009). These discrepancies in adult animals may relate with the physiological characteristics of the studied subjects. In the above-cited works, SPF young adult rabbits (20-weeks-old) or conventional primipregnant rabbit does (16 to 24-weekold) were used. In order to know the accumulative effect of successive gestation–lactation cycles in the immune system of high-performing selected rabbit does, the animals studied in this work were at the fifth lactation and sixth gestation stages and were older (11–15 months old). In their study with Angora rabbits aged 2.0–2.5 years old, Çetin *et al.* (2009) found that the total lymphocyte count vastly differed among males (5700×10^6/L), non-pregnant females (4300×10^6/L) and pregnant females on day 22 to 25 of gestation (2800×10^6/L), with the latest average being very similar to that obtained in this work with younger, but more biologically stressed rabbit does (high-performing selected animals that are either lactating or gestating, or both

simultaneously). In humans, the absolute count and the percentage of total lymphocytes have been described to decline with age (Hulstaert *et al.,* 1994). The lower lymphocytes count in the weaning rabbits of this study if compared with previous works (Jeklova *et al.,* 2007a, 2009) may relate with their mothers' immunological status. Maternal stress in pregnant sows has been reported to possibly induce long-lasting alterations in their offsprings' immunity (i.e., a decrease in lymphocytes) (Otten *et al.,* 2010). As indicated above, it may consider that the rabbit does of the present study were under greater productive stress than the SPF primiparous rabbit does used as parents in the aforementioned studies. Thus, it may be hypothesized that this fact affects their litters.

The $CD5^+$ lymphocytes count is higher than that of the B lymphocytes, while the $CD5^+$/B lymphocyte ratio is around 10 as an average of all the study animals, which is mainly due to very low B lymphocyte counts. A lower amount of B cells in peripheral blood has been reported as being relatively common in elderly persons (Franceschi *et al.,* 1995) and older cows (Ohtsuka *et al.,* 2009).

In general, the counts of total, $CD5^+$, $CD4^+$ and $CD8^+$ lymphocytes during lactation and at weaning are lower in the 42D does (insemination at 25 dpp and weaning at 42 dpp) than in the 28D ones (insemination at 11 dpp and weaning at 28 dpp). In addition, the 42D does are not apparently capable of adapting to the physiological status since no differences in these counts were detected throughout the experimental period. On the other hand, longer lactation periods and a presumable major wear of this group likely implies that the body condition of these animals becomes more relevant, to such an extent that the greater the PFT at partum, the higher the total and B lymphocytes counts around the top milk yield (16 dpp); furthermore, the greater the PFT at weaning or the lesser the PFT loss during lactation, the higher the $CD5^+$ and B lymphocytes counts at 1 week post-weaning. Milk yield requires great effort, even in its final period (Pascual *et al.,* 2003, 2006), and some studies have reported an improving of the body energy balance of females by shortening lactation duration (Xiccato *et al.,* 2004, 2005).

Conversely, the 28D does show an increased number of total lymphocytes at 28 dpp if compared to 16 dpp, which is followed by a decrease at 35 dpp and a further increase at 42 dpp. The evolution of the total lymphocyte count during pregnancy in rabbit does reaches a nadir on gestational days 22–24 (Wells *et al.,* 1999; Kim *et al.,* 2002b), i.e., around 35 dpp in this study.

Jeklova *et al.* (2007a) reported $CD4^+$/$CD8^+$ ratios of around 2.8 in SPF 4 to 20-week-old rabbits. In this study, similar results in adult rabbit does (average 2.75), and lower values in 28-day-old rabbits (2.32 ± 0.24) especially, 42-day-old rabbits (1.91 ± 0.22) were obtained, mainly due to a drop in $CD4^+$ lymphocytes.

As regards the relationship between females and weaning rabbits, the $CD5^+$ lymphocyte counts in females positively correlated with the $CD5^+$, $CD4^+$ and $CD8^+$ lymphocyte counts in young rabbits, but negatively correlated with the $CD25^+$ lymphocyte count. During human gestation, it has been reported that mothers acquire foetal lymphoid progenitors that develop into functional T cells (Khosrotehrani *et al.,* 2008). Besides, human milk contains numerous lymphocytes (1.5–3 $\times$ 10^5 cells/mL), of which 80% are T cells (Lönnerdal, 2000). These cells contribute not only to locally protect the intestine, but also to general passive cell

immunity as some can pass through the intestinal barrier and participate in the immunological reinforcement of young rabbits (Fortun-Lamothe and Drouet-Viard, 2002).

In conclusion, rabbit does with weaning at 42 dpp presented a lower number of total lymphocytes and lymphocytic subpopulations during lactation and at weaning, as well as lesser capacity of adjustment during the gestation–lactation cycle. This scenario might be related with longer lactation periods and major accumulated wear throughout their productive life under prolonged reproduction rhythms. This could imply a minor immunological level and them probably having a lower response capacity against infections. This fact apparently makes them depend on their body condition more than rabbit does with weaning at 28 dpp. In addition, their litters have a lower number of lymphocytes $CD4^+$, which are a fundamental part of immune response coordination.

Blood lymphocytes and their evolution from the first to the second parturition of rabbit does differing in animal type (by comparing two distant generations of a line selected for litter size at weaning and a line founded with reproductive longevity criteria) and the animals' response to heat stress in terms of animal type.

Often rabbit changes in blood count occur (Poljičak-Milas *et al.,* 2009). Referent haematological values in laboratory lagomorphs, the European rabbit included, have been described in literature on many occasions (Lepitzki and Woolf, 1991). However, rabbits display unpredictable oscillations in haematological values in particular in term of their lymphocyte populations under different challenges. The lack of information is more evident in commercial rabbits where it is vital to characterize and to evaluate rabbit genetic lines and their responses to environmental challenges, like heat stress, which is the case evaluated in the second part of this thesis.

By genetic selection, animals with different useful immunological characteristics in humoral or cellular immune responses can be obtained (Lavi *et al.*, 2005). Differences in the number of lymphocytes have been found between genetic lines of chickens (Cheeseman *et al.*, 2004) and between breeds of pigs (Clapperton *et al.*, 2005). Moreover, it has been proposed that these differences may be implied in resistance to infection by a wide range of pathogens and subsequent disease effects. However, as far as the authors are aware, such information is not available for rabbits. As previously reported (Wells *et al.*, 1999; Kim *et al.*, 2002b), the lymphocyte populations in the current study varied throughout the rabbit does' productive cycle, with differences found among the animal types involved.

Thus under conventional housing conditions, V36 females (generation 36th of the V line) showed lower counts at the second parturition than V16 females (generation 16th of the V line) for all the studied lymphocyte populations, with significant differences found for total, T $CD5^+$ and $CD25^+$, while differences in the B-lymphocytes were significant earlier (on 4 dpp and 10 dpp). It might hypothesise that selection for litter size at weaning might have some negative effect on the immune function. In this sense, PFT on 4 dpp was slightly lower in V36 females if compared with V16 or LP females (-0.25 mm, $P<0.10$) and a positive correlation was observed between PFT on 14 dpp and the B lymphocyte counts on 10 dpp. In a previous work done with females of the V line, Theilgaard *et al.* (2007) observed a higher risk of culling for rabbit does with a low fatness level on 10 dpp. However, litter size selection at weaning during 12 generations did not affect the risk of culling animals (Theilgaard *et al.*, 2006), but even increased the depth of PFT at 3 months of age in the more

selected animals (Quevedo *et al.*, 2005). Risk of culling in rabbit does peaks during the two first lactations, especially at the end of pregnancy (Rosell and de la Fuente, 2009); consequently, the possible relationship of the differences found in lymphocyte counts and the culling rate (through illness or death) due to litter size selection at weaning deserves further research.

LP females showed similar counts to V36 females during the study period, although a sharp drop in the total, $CD5^+$, $CD4^+$ and $CD8^+$ lymphocytes was observed from parturition to 4 dpp in LP females, but not in V36 females; besides, the $CD25^+$ counts were higher for LP females than for V36 females on 4 dpp, 10 dpp and at the second parturition. A higher level of T-activated cells may evidence a stronger robustness of LP females as opposed to V36 females. Previous studies have demonstrated major robustness for LP rabbit does if compared with the animals from the 31st generation of the V line; this has been related to a more efficient utilisation of their body reserves to successfully confront environmental (heat stress and feed restriction)or productive challenges (Theilgaard *et al.*, 2007).

On the other hand, immune cells of different animal species are affected by high temperatures. Thus, there have been reports of a fall in splenic NK cell activity in mice (Won and Lin, 1995), a smaller number of lymphocytes in the spleen, mesenteric and peripheral lymph nodes in rats (Krynicki and Olszewski, 1989), and enhanced lymphocyte quantity in the bone marrow of limbs and spine in rats (Krynicki and Olszewski, 1989). Besides, chronic heat stress impairs the expression of contact sensitivity in vivo and the proliferation of T lymphocytes in vitro in avian species, although the B-cell and T-helper cell functions were not compromised (Regnier and Kelley, 1981). However, Franci *et al.* (1996a) reported how thermal stress treatments diminished the capacity of rabbits' peripheral blood mononuclear cells to proliferate and inhibit the differentiation of B lymphocytes in antibody-secreting cells, which induce a suppression of either immunoglobulin production or IL-2 synthesis (Franci *et al.*, 1996b). Besides, lymphocytes' resistance to heat stress has been reported to be modified by breed in chickens (Regnier and Kelley, 1981) and bovines (Kamwanja *et al.*, 1994).

In the present study, heat stress also differently affected the lymphocyte populations of the compared rabbit does as the differences observed under conventional housing between the V36 and V16 populations (in the B lymphocyte counts on 4 dpp or 10 dpp, and in the total, $CD5^+$ and $CD25^+$ lymphocyte counts at the second parturition) and those observed at the second parturition generally increased to favour LP females if compared to V36 females under heat stress conditions (especially total lymphocyte counts), except that observed for $CD25^+$. So it can be hypothesised that the former finding could be related to V populations being selected in a warm climate (Spanish Mediterranean) and/or to there being no differences in the body condition between V16 and V36 when housed under heat stress conditions. This fact suggests that litter size selection at weaning may not affect females' immune function under heat stress conditions. This last finding might contribute to the above-mentioned robustness of LP line rabbits if compared to V populations, resulting in a lower risk of culling and longer productive life (Sánchez *et al.*, 2008).

In conclusion, these results indicate that, under conventional housing conditions, litter size selection at weaning for 20 generations may affect the immune system since V36 animals had lower lymphocyte counts than V16 animals at a very critical time (e.g., the second parturition), whereas under heat stress conditions, the animals from a line founded by

screening for reproductive longevity (the LP line) showed higher lymphocyte counts at this particular stage than those from V36. This scenario could contribute to a greater ability to confront infectious challenges and to confer animals a more robust nature.

Effect of selection of rabbit does for either longevity and litter size or litter size at weaning on their immune responses to lipopolysaccharide.

Bacterial infections are accompanied by potent host responses that are often followed by opposing anti-inflammatory effects (Lewkowicz *et al.,* 2006). LPS, the primary toxic component of endotoxin, located in the cell wall of Gram-negative bacteria, elicits a complex acute phase response (Krueger and Majde, 1990), which can be followed by fever and changes in the blood concentrations of some physiological metabolites and acute phase proteins. Although different studies have evaluated the response in rabbits after moderate (<5 mg/kg BW; Kimura *et al.,* 1994; Amador *et al.,* 2007; Huang *et al.,* 2008; Marca *et al.,* 2009) or severe LPS challenges (50 to 85 mg/kg BW; Mathison and Ulevitch, 1979; Saitoh *et al.,* 1999, 2000) at hormonal, cellular and tissue levels, knowledge related to other factors affecting the immunological response is scarce.

The fever induced after challenge (between 0 and 3 h pi) is a normal adaptation in response to the LPS pyrogenic stimulus, that leads to a proportional rise of plasma glucose content (Kiviranta *et al.,* 1995) but without associated changes in NEFA level. Increased glucose utilization to support immune system functions and reduced liver fatty acid oxidation just after infection has been widely described (Blackburn, 1977; Grunfeld and Feingold, 1992). This has also been observed in rabbits, where the maximum temperature is reached between 1.5 to 3 h pi both in mild (Huang *et al.,* 2008; Shibata *et al.,* 2005; Kimura *et al.,* 1994) and severe inductions (Saitoh *et al.,* 2000), with recovery to the normal ranges from 24-48 h pi. The rectal temperature peak is reached in cows and sheep about 4 h after LPS inoculation (Waggoner *et al.,* 2009; Yates *et al.,* 2011) with an increase of plasma glucose content at 2 h pi as a result (Waggoner *et al.,* 2009; Stengel *et al.,* 2010; Bernhard *et al.,* 2012).

However, a subsequent decrease of the plasma glucose level was observed from 6 to 24 h pi just as NEFA levels began to rise. LPS induction usually drives the liver to atotal depletion of hepatocyte glycogen. This, together with the concomitant lack of feed consumption, might lead to the pronounced hypoglycemia (Ferrante *et al.,* 1984; Fukuzumi *et al.,* 1996; Leininger *et al.,* 2000) and NEFA mobilization (Webel *et al.,* 1997; Leininger *et al.,* 2000; Kushibiki *et al.,* 2009), commonly observed after LPS challenges in other species.

Another systemic response to disease is an increase in liver production of acute-phase proteins (Jain *et al.,* 2011), as was observed in the present study from 6 h pi, and in previous works with rabbits (Murray and Connel, 1960; Mackiewickz *et al.,* 1988; Baker and Long, 1990; Petersen *et al.,* 2004; Georgieva *et al.,* 2009). Haptoglobin plays both an antioxidative (Carter and Worwood, 2007) as well as a bacteriostatic role by restricting the free iron needed for bacterial growth (Eaton *et al.,* 1982), while C-reactive protein has the ability to increase opsonization and activation of the complement system (Petersen *et al.,* 2004). Therefore, the greater the increase of these proteins in the blood, the greater was the response against the infectious agent.

Although both lines have shown a very similar response pattern for most of the parameters measured after LPS challenge, punctual differences between them should be highlighted. LP

females showed an earlier drop of maximum temperature and lower glucose release during the initial acute phase, as well as higher amount of plasma C-reactive protein and especially haptoglobin after 24 h pi, with respect to V females (**Figures 61** and **63c, d**). The lower early acute reaction of LP females (first 6 h pi) coincided with the period where the gap in survival between the genetic lines occurred (+20% at 6h pi; $P<0.05$). This gap was subsequently maintained. In rabbits (Kluger and Vaughn, 1978), as in other species (Kluger *et al.,* 1975; Reynolds *et al.,* 1976; Bernheim and Kluger 1977), an initial positive correlation between the fever magnitude and survival has been reported, although the correlation was reversed when the fever was too high during acute challenges, with high and maintained temperatures increasing the risk of death. In this study, results showing genetic differences during the early severe phase of the challenge, leading to longer survival for those animals showing a lower acute response at this critical stage followed by a higher later response to the inoculum via increased acute phase proteins, indicating clear differences in the ability to mount an immune response to the LPS challenge.

Thus, the consequences of the criteria used during the foundation or selection, on the ability of the females to confront immunological challenges, seems to lie in the differences between the studied lines in resource allocation. From the correlations reported in **Table 14**, it might be deduced that the better the body condition of females before challenge, the lower the acute impact of LPS (lower rise of the rectal temperature and glucose release to plasma), the lower the mobilization of reserves via NEFA, and the higher the response after inoculation of LPS via acute phase proteins. Further, the only blood trait correlated with survival was NEFA (**Table 16**). In that respect, the first study of this thesis (Study I) have indicated that the greater was the body condition of rabbit females the greater their lymphocyte count, and that rabbit does in a good body condition showed a lower risk of culling or death (Theilgaard *et al.,* 2006). These results may lead to hypothesize that body reserves might actively participate in the modulation of the immune system response.

Rabbit females from the LP line have been characterized by a longer reproductive life than females only selected for reproductive traits (Sánchez *et al.,* 2008). This difference is associated with their greater soma (body weight and body condition) at the beginning of their reproductive life and their greater robustness in productive and environmental challenges (Theilgaard *et al.,* 2007, 2009; Savietto *et al.,* 2013a). Pascual *et al.* (2012) proposed that the nutrient partitioning capacity of these robust females enabled them to better cope with the possible reproductive, environmental, and probably immunological challenges that they might meet in the course of their productive life. This might explain their higher life expectancy on the farm. In fact, the Study II of the present thesis, comparing the lymphocyte population in blood of rabbit does from the 36th generation of line V with that of does from line LP, showed higher lymphocyte counts and better responses under heat stress conditions for LP females. It must be considered that the robustness definition refers mainly to health, and as proposed by Ellen *et al.* (2009), robust animals must be less sensitive to disease and their immunological response must allow a quicker recovery than less robust animals. This was the case in the present study.

Finally, our results showed that the advantage in terms of survival rate of the LP over the V line came exclusively from females mated after first weaning (PW; **Table 15**), which were also the main group responsible for the increase of NEFA in blood after 6 h pi (**Figure 62**). Females mated just after first parturition (PP9) did not show genetic differences on survival rate or increased NEFA blood level during the challenge. This difference should be

interpreted carefully since the PP9 group was set up from females successfully pregnant just after first parturition, whereas the PW groups were constituted from those does that failed to become pregnant just after first parturition. It can be hypothesized that this mating procedure could have performed a pre-selection for especially robust rabbit does. In fact PP9 does were characterized by lower mortality rate during the challenge, independently of their genetic type.

In conclusion, a selection line (LP) founded using rabbit does with exceptional productive hyperlongevity was found to be more robust with respect to health than a line selected only for reproductive intensity. The LP does showed lower sensitivity to challenge with bacterial LPS, make them more robust type of animals may also have greater capacity to withstand immune challenges, and that the introduction of this type of animals might contribute to maintaining an adequate productive level and health in commercial rabbit farms.

FINAL CONCLUSIONS

F. FINAL CONCLUSIONS

From the results of the three studies that this thesis comprises, it can be concluded that there seems to be an evident relationship between reproductive rabbit does' body condition and health status as clear positive correlations have been found between body condition and main lymphocyte population counts in the first two studies, and NEFA levels in blood seem to be the only plasma trait relating to female survival in the immunological challenge developed in the third. Thus, these results confirm the initial hypothesis of this thesis; that is, that body condition is proposed to play a mediating role which, for animals at the same production and genetic level, entails: the better the body condition, the better the immune response.

In line with this, the first thesis study reveals that there is a relationship between the lymphocytes populations of rabbit mothers and those observed for their litters (mainly some T lymphocyte subsets), and that those females with less physiological wear are more capable of modulating their lymphocyte populations in a less body condition dependent-way. Therefore before adopting reproductive management systems, which could increase female physiological wear (e.g., prolonged lactations), their possible added effects on rabbit females' immune response ability and on defining their litters' health status during the ulterior growing period must be considered. However, additional further research into this relationship must be enhanced to define possible global effects in the future.

Within the frame of these new global breeding systems to improve total farm health status, genetic type of animals could prove a relevant factor. The results of the second study show how selection for high reproduction (litter size at weaning) seems to lower lymphocyte counts during some high exigent periods of the reproductive cycle (B Lymphocytes at lactation and $CD5^+$ at second parturition), which could evidence possible negative effects of this selection criteria on female rabbits' immune function. In fact, when compared with a line founded for reproductive longevity criteria, and distinguished for greater robustness (less sensitivity to environmental changes), robust females were characterized by unaffected counts on lymphocytes under normal environmental conditions.

However, environmental conditions sporadically deviate from these normal conditions (such as heat stress frequently observed in summer on rabbit farms), which worsen females´ general immune status. Selection for litter size at weaning may not affect females´ immune function under these heat stress conditions, but robust females are characterized by their better ability to adapt their immune system under these challenging conditions. In fact, the results of the third study highlight that these robust females submitted to a sporadic infectious challenge show higher survival rates and acute phase responses, thus conferring them a greater ability to withstand immune challenges.

Therefore, it could be concluded that, when environment and reproductive management ensures sufficient resources to avoid body condition being jeopardized, selection for reproductive traits (such as litter size at weaning) can improve economic traits without jeopardizing animals' immune status. However, when resources become limited, the preferential allocation of resources in selected traits (such as reproduction) could diminish rabbit females' ability to respond to other demands (such as coping with disease). As farms are frequently subjected to sporadic challenges (heat stress, pathogens, etc.), the particular nutrient partitioning of females characterized by higher robustness could contribute to maintain an adequate productive level and health on commercial rabbit farms.

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G. REFERENCES

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